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FAK-regulated IL6 reprograms the tumour associated macrophage phenotype and promotes pancreatic tumour growth

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Declaration

I declare that this thesis has been submitted by myself, describes my own work and has not been submitted in any other application for a higher degree. Where others have contributed to work described in this thesis, this is indicated by clear citations or statement of contribution.

Catherine Davidson
August 2018

Dedication

This thesis is dedicated to my wonderful dad, John Maxwell Davidson, who died before my PhD began, but who has and always will be an inspiration.

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It feels like only yesterday that I embarked upon my PhD, but alas, three years have passed and it has come to an end. I have thoroughly enjoyed my time and I am grateful for all I have learned and for those who have taught me. None of this would have been possible without the support and encouragement of a great many people whom I shall endeavour to thank here.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a cancer with few effective therapeutic options, and patient prognosis is poor. Immunotherapy has yet to yield significant benefit in treatment of PDAC. Understanding the key molecular pathways that drive resistance to immunotherapy may enable development of new therapeutic strategies for this cancer of unmet clinical need. The non-receptor protein tyrosine kinase, Focal Adhesion Kinase (FAK), is up-regulated in PDAC, and recent studies have identified an important role for FAK in regulating the fibrotic and immunosuppressive pancreatic tumour microenvironment (TME). However, the mechanisms underpinning FAK-dependent regulation of the immunosuppressive TME remain poorly understood. Using CRISPR, I have depleted FAK expression in pancreatic cancer cells isolated from PDAC arising on *LSL-Kras*$^{G12D/+}$*;LSL-Trp53*$^{R172H/+}$*;Pdx-1-Cre* mice (KPC mice), and show that FAK-depletion results in a tumour growth delay that is associated with reprogramming of the tumour associated macrophage (TAM) phenotype. Mechanistically I identify FAK-dependent regulation of interleukin-6 secretion from pancreatic cancer cells as an important regulator of the TAM phenotype, and further show that this axis requires $CD4^+$ T-cells. These results provide new insights into the complexity of FAK-dependent immune regulation in cancer, and support continued evaluation of FAK kinase inhibitors in combination with immunotherapy for the treatment of PDAC.

Lay summary

Pancreatic cancer patients currently face a very poor prognosis. More than 95% of patients die within 5 years of diagnosis. There are currently no effective long-term treatments for patients who have been diagnosed with advanced disease and are ineligible for surgery, a situation representing the majority of pancreatic cancer patients.

Many tumour types including pancreatic cancer are made of up lots of different types of cells. Cancer cells talk to the other surrounding cells and manipulate them to aid cancer cell multiplication. Tumour associated macrophages are an example of one of these cell types. These can be manipulated by signals derived from cancer cells to become one of two types: M1 or M2, and by using markers on the surface of these macrophages, we can identify them within pancreatic tumours. The two types of macrophages have very different functions within tumours. M2 macrophages promote tumour progression whereas M1 macrophages help attack tumour cells. By manipulating macrophages within the tumour to the M2 type, this is one way in which pancreatic tumour cells hide from the immune system. This is termed 'immune evasion'.

Immunotherapy works by stimulating the body's immune system to recognise and attack the cancer cells. By inhibiting a protein called Focal Adhesion Kinase (FAK) within the pancreatic tumour cells, I have discovered a way of inhibiting the ability of cancer cells to talk to the macrophages within the tumour and cause them to take on the M2 type. I show that this allows other cells that make up the immune system better able to destroy pancreatic tumours. The results of this study suggest that by using drugs that inhibit the FAK protein in pancreatic cancer, this potentially leads to a more effective treatment response and could be useful as part of the treatment regime in this disease.

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List of Abbreviations

α-SMA	alpha-smooth muscle actin
ACT	Adoptive cell transfer
ADEX	Aberrantly differentiated endocrine-exocrine
ADP	Adenosine diphosphate
APC	Antigen presenting cell
ATP	Adenosine triphosphate
c-maf	V-maf musculoaponeurotic fibrosarcoma oncogene
CAF	Cancer associated fibroblast
CAR	Chimeric antigen receptor
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CM	Conditioned media
cDNA	Complementary deoxyribonucleic acid
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXC	C-X-C motif chemokine
CSF1/R	Colony-stimulating factor 1 / receptor
DC	Dendritic cell
DcR3	Decoy receptor 3
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
effTreg	Effector regulatory T cell
ELISA	Enzyme-linked immunosorbant assay
FA	Focal adhesion
FACS	Fluorescence-activated cell sorting
FAT	Focal adhesion targeting
FERM	four-point-one, ezrin, radixin, moesin
FCS	Foetal calf serum
Foxp3	Forkhead box P3
GATA-3	GATA binding protein 3
GMDSC	Granulocytic myeloid-derived suppressor cell
GM-CSF	Granulocyte-macrophage colony stimulating factor
GP130	glycoprotein 130
Grb7	Growth Factor receptor bound protein 7
GTP	Guanosine triphosphate
H&E	Haematoxylin and eosin
HLA-Dr	Human leukocyte antigen- antigen D related
HBSS	HANKS balanced salt solution
i.p.	Intraperitoneal
i.v.	Intravenous
ICS	Intracellular cytokine staining
ICOS	Inducible T-cell co-stimulator
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon-γ
IHC	Immunohistochemistry
IL-	Interleukin-
IL1RL1	Interleukin 1 receptor-like 1, or ST2

IP_3	Inositol-triphosphate
IPMN	Intraductal papillar mucinous neoplasms
JAK	Janus kinase
KDM6A	Lysine-specific demethylase 6A
KO	Knockout
KRAS	Kirsten rat sarcoma viral oncogene homolog
LB	Lysogeny broth
LAG-3	Lymphocyte-activating gene 3
LMP	Latent membrane protein
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MCN	Mucinous cystic neoplasm
M-MDSC	Monocytic myeloid derived suppressor cell
MHC	Major histocompatibility complex
Min	Minutes
MMP	Matrix metalloproteases
MMR	macrophage mannose receptor
MUC-1	Mucin-1
mRNA	Messenger ribonucleic acid
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T-cells
NFκβ	Nuclear factor kappa β
NK	Natural killer
NKG2D	Natural killer group 2, member D
NLS	Nuclear localisation sequence
NOS	Nitric oxide synthase
PanIN	Pancreatic intraepithelial neoplasia
PaSC	Pancreatic stellate cells
nTreg	Natural Treg
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
PD-1	Programmed death-1 receptor
PDL-1/2	Programmed cell death ligand-1/2
PDGF	Platelet derived growth factor
PI3K	Phosphoinositide 3- kinase
PR	Proline rich
RBC	Red blood cell
ROI	Reactive oxygen intermediates
Retnla	Resistin-like molecule alpha
RT	Room temperature
RORγT	RAR-related orphan receptor gamma
s.c.	Sub-cutaneous
SCC	Squamous cell carcinoma
SMAD4	Mothers against decapentaplegic homolog 4
SOCS	Silencer of cytokine signalling
ST2	Suppression of tumorigenicity
STAT	Signal transducer and activator of transcription proteins
TAM	Tumour associated macrophage

TAP	Transporter associated with antigen processing
T-bet	T-box transcription factor TBX21
TP53	tumour protein 53
TCR	T-cell receptor
Teff	Effector T cell
Tie2	Tyrosine protein kinase receptor-2
TIL	Tumour infiltrating lymphocyte
TGF-β	Transforming growth factor β
T_H	T helper
TIM-3	T-cell immunoglobin and mucin domain-3
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor α
Treg	Regulatory T-cell(s)
wt	Wild-type
VEGF	Vascular endothelial growth factor
5-FU	Fluorouriacil

1 Introduction

1.1 Pancreatic cancer

1.1.1 Defining the problem

Despite our increased understanding of the biology of pancreatic cancer, major clinical advances have not been forthcoming. Pancreatic cancer is currently the fourth leading cause of cancer death in both the US[1] and Europe[2], and is expected to become the second most common in the developed world within the next decade[3], making this an increasingly lethal cancer type. Hence there is a real and urgent need to identify new strategies for the treatment of pancreatic cancer.

1.1.1.1 Epidemiology

Approximately 9,800 pancreatic cancer diagnoses are made in the UK every year, making pancreatic cancer the 11th most common cancer, accounting for 3% of all newly diagnosed cancer cases (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer). Over the last decade the incidence has increased by 11% and this trend is expected to continue (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer). There are several factors contributing to this rising trend. Pancreatic cancer has a strong association with increasing age[4] and therefore the global burden of this disease will likely increase due to rising population longevity. Additionally, the incidence of pancreatic cancer varies greatly between different countries with reported incidence higher in developed countries[5]. Although the number of cases reported in some countries could be attributed to better access to advanced diagnostics, generally this geographical distribution of cases reflects recognised socioeconomic risk factors such as obesity, diabetes, dietary factors, alcohol consumption, cigarette smoking, and pancreatitis[4], all playing a role in the aetiology of this disease. The relationship between chronic pancreatitis and pancreatic cancer is well established[6]. Although this relationship is difficult to quantify, as individuals with pancreatic cancer also suffer episodes of pancreatitis as a consequence of their tumour, it is reported that patients with chronic pancreatitis have a 13.3 fold increased relative risk of developing pancreatic cancer[7]. The relationship between chronic inflammatory insult with neoplastic development and progression is well recognised, and is thought to be influenced by inflammatory

cytokines[6], however the higher incidence of chronic pancreatitis compared to pancreatic cancer and the lack of pancreatitis in the clinical history of some cancer patients would suggest that inflammation is only part of the disease process.

The genetic alterations underpinning pancreatic tumourogenesis and progression are now well defined, and will be described in more detail in later sections of this chapter, however the genetic basis underlying familial pancreatic cancer remains controversial[8]. A family history of pancreatic cancer considerably increases an individual's risk of developing the disease, with 10% of cases reported to have a familial basis[8]. In a study of 102 familial pancreatic cancer patients, three mutations in the *BRACA2* gene and one mutation in *BRACA1* and *p16* were identified[9].

1.1.1.2 Survival

Pancreatic cancer has an extremely poor prognosis with a 5-year survival rate of less than 5%[1,10] and a 10- year survival rate of less than 1% in the UK. There has been little improvement in the survival rate of pancreatic cancer patients over the last 40 years (http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer/survival), highlighting a clear and urgent unmet clinical need. This poor survival rate is thought to be due to a variety factors, mainly attributed to aspects of pancreatic tumour biology, resulting in late diagnosis, and a poor response to treatment. The majority of patients have aggressive and metastatic cancer at the time of diagnosis[11,12] due to metastasis occurring early in the disease course and clinically silent disease until advanced stages[13].

1.1.1.3 Current treatment options

Poor clinical response to current therapeutic regimes and the lack of substantive advancement in effective new treatments are major issues in treatment success of pancreatic cancer. Surgical resection is at present the only treatment modality to significantly prolong survival times. Unfortunately the clinically silent nature of this disease in the early stages, coupled with the lack of early detection methods, results in diagnosis often being made after the tumour has invaded surrounding tissues or spread to distant sites. For this reason only approximately 15% of patients are eligible for curative intent surgery[14]. Even in those patients who are suitable surgical

candidates, there still remains only a modest survival benefit of 14-20 months[15] due to a high rate of recurrence. Neoadjuvant chemotherapy or adjuvant chemotherapy following surgery is often employed. Although a number of randomised controlled trials demonstrate a significant survival advantage in patients treated with adjuvant chemotherapy after resected pancreatic tumours and neoadjuvant gemcitabine-radiotherapy regimes[8,14], a significant overall survival benefit was not established in surgical patients receiving neoadjuvant or adjuvant therapy from recent meta-analysis data[15].

Once the tumour has locally advanced into surrounding structures or spread to distant sites, palliative chemotherapy is often considered. Currently the standard of care for most patients with advanced pancreatic cancer is gemcitabine-based regimens either as a single agent or more recently in combination with nab-paclitaxel, a nanoparticle albumen-bound form of paclitaxel, giving mean survival benefit of 1.8 months compared to gemcitabine alone[16], or aggressive multi-drug regimes such as FOLFIRINOX, a multi-drug combination of leucovorin, fluorouracil, irinotecan and oxaliplatin, giving a mean survival benefit of 4.3 months compared to gemcitabine alone[17]. These survival benefits can arguably be regarded as only incremental at best and therefore justify the importance of further research to identify new strategies with the potential for long-term clinical responses for this devastating cancer type.

1.1.2 Pancreatic cancer biology and molecular pathology

Pancreatic cancers are classified according to the cell type they arise from with the majority developing from the ductal lineage. Pancreatic ductal adenocarcinoma (PDAC) is the predominant histological type of pancreatic cancer, occurring in 90% of cases[18]. Less common non-ductal pancreatic cancers include: fibrous histiocytoma, juvenile haemangioendothelioma, schwannoma, leiomyosarcoma, malignant schwannoma, fibrosarcoma, malignant fibrous histiocytoma, liposarcoma, rhabdomyosarcoma, malignant haemangiopericytoma, extramedullary plasmacytoma and neuroendocrine tumours arising from the islets of Langerhan.

PDAC arises from various precursor lesions, in most cases from microscopic non-invasive epithelial proliferations within the pancreatic ducts, referred to as pancreatic intraepithelial neoplasias (PanINs)[19]. Less commonly, PDAC can also arise from larger macroscopic cystic precursors namely mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs)[20].

The precursor lesions (PanIN-1, 2 and 3) have been traditionally categorised on the basis of increasing cytological and architectural disorganisation. PanINs have been described as ducts measuring less than 5mm in diameter and multiple stages can occur in the same pancreas, displaying increasing cytoarchitectural atypia as lesions progress from PanIN-1 to PainIN-3[21]. PanIN-3 demonstrates severe nuclear and cellular atypia and can be considered 'carcinoma in-situ'[22]. These eventually develop into invasive carcinoma with a marked stromal reaction and ultimately into disseminated metastatic disease (**figure 1.1**). More recently an international consensus meeting has recommended a two-tiered classification system to replace the original three-tiered classification. This low-grade verses high-grade classification system is based on histological appearance and is thought to better reflect the clinical significance of the lesions[23]. In addition to the ductal carcinogenesis sequence, two alternative precursors pathways of invasive carcinoma have also been proposed, namely acinar-ductal metaplasia (ADM)[24] and atypical flat lesions (AFLs)[25], however their clinical and biological significance requires further research[23].

The cytological changes described in the step-wise progression from PanIN-1, through PanIN-2 to PanIN-3 are accompanied by progressive accumulation of well described genetic alterations as shown in the adapted figure below (**figure 1.1**) from Bardeesy et al[26]. The process begins in early PanIN-1 with telomere shortening and activation of the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS)* oncogene, most commonly an activating point mutation in codon 12[27–29]. *KRAS* is involved in the regulation of cell division as a result of its ability to relay external signals to the cell nucleus. Activating mutations in the *KRAS* gene result in a permanently active KRAS protein, and resultant downstream pathway activation leads to cell transformation[30]. *KRAS* is mutated in over 90% of all PDACs[18,31,32] and the remainder of PDACs that do not harbour *KRAS* mutations tend to exhibit de-

regulated *KRAS* signalling through mutations of other genes in the *KRAS* signalling pathway, suggesting that *KRAS* and /or pathway mutations are critical initiating genetic alterations in this disease[31–33]. Loss of function mutations in tumour suppressor gene *CDKN2A,* a gene encoding p16, a protein playing an important role in regulating the cell cycle, is also a frequent early event[31]. PanIN-2 and PanIN-3 often acquire additional loss of function mutations in the tumour suppressor genes *TP53* (tumour protein 53) and *SMAD4* (Mothers against decapentaplegic homolog 4), that drive progression towards PDAC[34]. Mutations in *TP53*, a key protein involved in the cellular stress response, and *SMAD4*, a gene mediating signalling downstream of transforming growth factor β (TGFβ), occur in greater than 50% of cases[35]. In addition to these four driver gene mutations, recent data from whole exome and whole genome sequencing on expansive sets of patient derived tumour samples have revealed a large number of additional, less frequent gene mutations, exposing the complex mutational landscape underlying pancreatic tumourogenesis[32,35,36]. These studies have greatly increased our understanding of additional genetic alterations underlying tumourogenesis in pancreatic cancer and furthermore have identified subtypes of pancreatic cancer. These subtypes are categorised based on mutational signatures, accompanying histological changes and differences in patient survival, inferring distinctive mechanisms underpinning molecular evolution. The four proposed subtypes are: 1) squamous, with increased levels of *TP53* and *KDM6A* (Lysine-specific demethylase 6A) mutations, up-regulation of the TP63ΔN transcriptional network and down-regulation of pancreatic endodermal cell-fate determining genes resulting in loss of endodermal identity; 2) the pancreatic progenitor or classical PDAC subtype which expresses early pancreatic development genes (i.e., *PDX1, FOXA2/3, MNX1*); 3) aberrantly differentiated endocrine/exocrine (ADEX) tumours, which overexpresses genes involved in KRAS activation and those in later stages of pancreatic development such as exocrine (NR5A, RBPJL) and endocrine (NEUROD1, NKX2-2) markers; and 4) immunogenic PDAC. Immunogenic PDAC has molecular resemblances to the progenitor/ classical PDAC subtype but also contains over-expressed genes associated with infiltration of immune cells. For example increased expression of genes associated with $CD4^+$ T-cells, $CD8^+$ T-cells and immune network pathways including those associated with acquired immune suppression and immune evasion such as up-regulation of the

immune checkpoint molecules CTLA4 and PD-1[36]. Categorising patients into these subtypes may be of clinical relevance. For example, patients with the immunogenic PDAC subtype may represent suitable patients for immunotherapy and therefore subtypes may provide new opportunities for personalised medicine treatment strategies.

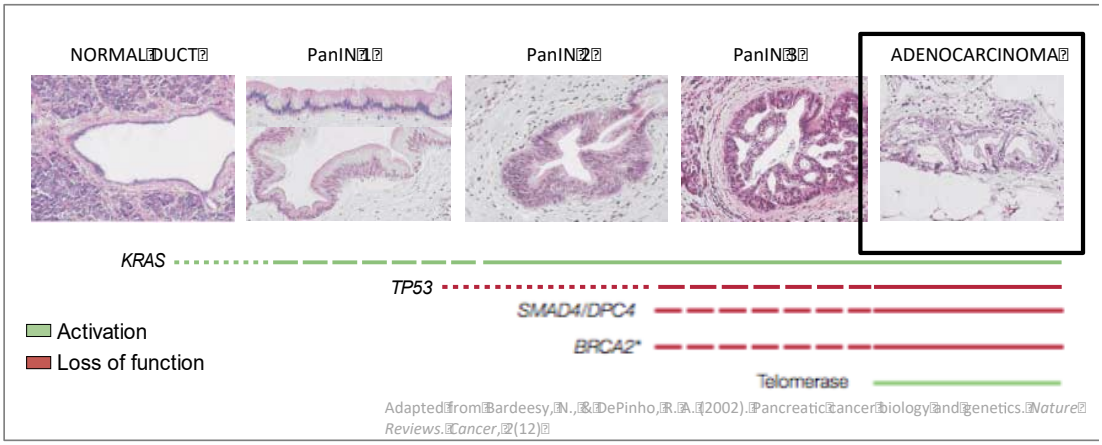


Figure 1.1 Histological progression from normal epithelium through pancreatic intraepithelial neoplasia (PanIN 1, 2 and 3) and to pancreatic adenocarcinoma.

Images of histological progression depict increasing cell dysplasia, nuclear abnormalities and local invasion. *KRAS* mutations increase in frequency as the disease progresses, as does frequency of other mutations such as, but not limited to, *TP53* tumour-suppressor gene mutations. *KRAS* mutations are generally found in early stage disease whereas *TP53* mutations arise in later stages. Cells derived in this study are derived from LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$ pancreatic cancer cells at the adenocarcinoma stage.

1.1.3 The pancreatic tumour microenvironment

Components of the tumour microenvironment (TME) are now known to be key factors in tumour growth and development. Cancer cells themselves are instigators of the disease and the driving force behind tumourogenesis, but the cancer cell's ability to manipulate the surrounding TME components is crucial to tumour survival[37,38].

A highly fibrotic and immunosuppressive TME is characteristic of PDAC and occupies the majority of the tumour mass[39–41]. This pronounced stromal compartment

(**figure 1.2**) is comprised of cellular and acellular elements: immune cells, fibroblasts, myofibroblasts, pancreatic stellate cells, endothelial cells, extracellular matrix (ECM) and soluble proteins such as cytokines and growth factors. The pancreatic tumour stroma is thought to play a major part in poor treatment response and resistance to therapy[42] through supporting tumour growth and metastasis and also acting as a physical barrier to penetration of cytotoxic drugs[39,43–45].

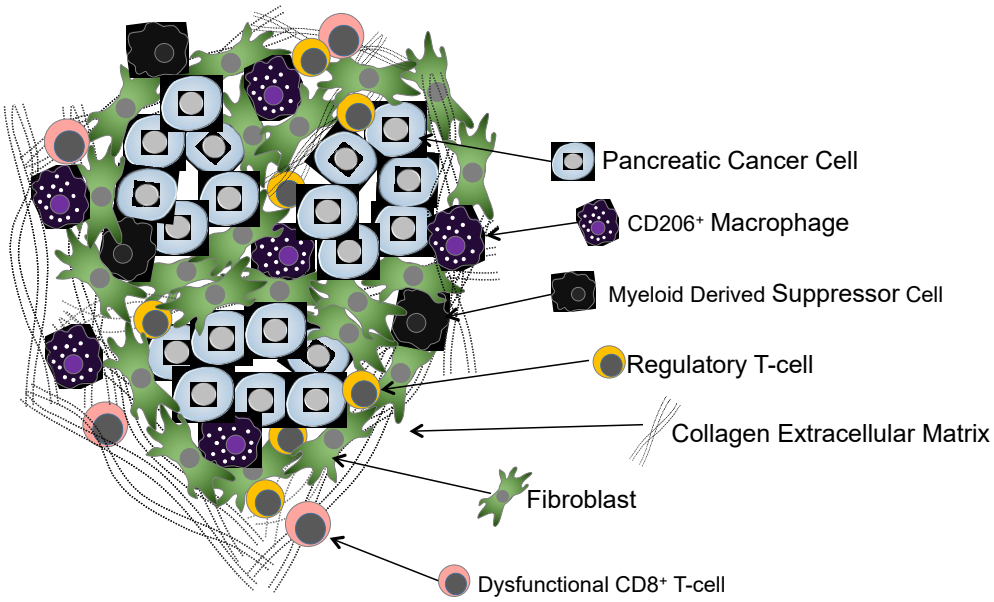


Figure 1.2 Diagram illustrating the components of the immunosuppressive pancreatic tumour microenvironment

1.1.3.1 Immune cells within the pancreatic TME

It is becoming increasingly apparent that inflammatory cells within the TME are recruited to help rather than hinder tumour cell growth[46,47]. Cancer cell ability to hijack the TME immune cell compartment in order to escape the immune response is now recognised as one of the key hallmarks of cancer[37]. Immune cell types within the pancreatic TME are mainly of the immunosuppressive type and include: macrophages, myeloid-derived suppressor cells (MDSCs), regulatory T-cells (Tregs)

and low numbers of $CD8^+$ T-cells[48,49]. The presence of immunosuppressive cell types from the earliest of PanIN lesions which increase in frequency through progression to carcinoma is suggestive of the importance of such cell types in progression of this tumour from the earliest stages[50].

1.1.3.1.1 Tumour associated macrophages in PDAC

Macrophages are mononuclear phagocytes playing a central role in tissue homeostasis and immunity. Under normal inflammatory conditions, monocytes localise to sites of inflammation in tissues, after which they are termed macrophages and act to fight infection or mediate wound healing through release of growth factors and cytokines[51]. The most abundant inflammatory cell type in the majority of solid tumours are macrophages and can contribute up to 50% of the total tumour mass[47,52]. PDAC is an example of one of these tumour types and is very heavily infiltrated by tumour associated macrophages (TAMs)[53]. TAMs are comprised of two major macrophage subtypes: resident and inflammatory macrophages[54]. In PDAC these are largely derived from embryonic and bone marrow derived (BMD) sources respectively[55,56]. Macrophages are highly plastic cells and can be polarised to opposing phenotypes: M1 (classical) and M2 (alternative). Classical activation occurs in response to T-helper 1 (T_H1) cytokines, for example interferon γ (IFNγ) and Toll-like receptor (TLR)-ligands such as lipopolysacharide (LPS). In infectious disease, macrophages are activated to the M1 phenotype to fight intracellular pathogens, and in cancer drive an anti-tumour response[57] by secreting tumouricidal factors such as tumour necrosis factor α (TNF-α), interleukin-12 (IL12) and reactive oxygen intermediates (ROI)[42]. M1 macrophages can be identified in various ways such as expression of 'M1 genes': *IL12b, Ciita* (Class II Major Histocompatibility Complex Transactivator) and *Nos2* (nitric oxide synthase)[54,58] and increased expression of CD86 and major histocompatibility complex class II (MHC-II)[59]. In contrast, alternative macrophage activation to the M2 phenotype occurs in response to T_H2 cytokines[60]. T_H2 derived IL4, in addition to Treg derived IL10 is linked to the induction of pro-tumour macrophage polarisation. It is also thought that T_H2 derived IL13 may have an overlapping role with IL4 as both their resultant signalling cascades lead to signal transduction and transcription (STAT) 6 transcription[61]. T_H2

cells then release immunosuppressive cytokines such as IL10, IL6 and TGF-β[62] into the TME.

M2 macrophages promote wound healing through activation of fibroblasts, angiogenesis, matrix remodelling and reducing inflammation[60,63]. These mechanisms support tumour growth in the context of cancer, hence M2 macrophages are regarded as having a pro-tumour phenotype. M2 macrophages can be identified by increased expression of 'M2 genes' such as *MMR* (macrophage mannose receptor, also known as CD206)*, Tie2* (Tyrosine-protein kinase receptor Tie-2)*, Arg1* (arginase 1) and *Retnla* (Resistin-like molecule alpha) and have increased surface expression of the mannose receptor (MMR/CD206), reduced CD86 expression, reduced MHC-II expression[59] and are therefore poor antigen presenters. M2 macrophages also express immune checkpoint ligands such as programmed death ligand-1 and 2 (PD-L1 and PD-L2)[60] which bind to the programmed cell death-1 (PD-1) receptor on the surface of $CD8^+$ T-cells, inhibiting T-cell function through T-cell anergy or death. In pancreatic cancer, the majority of TAMs are of the M2 phenotype[60,64] and a higher M2:M1 ratio is associated with poorer survival[48]. Additionally, peripheral accumulation of M2 macrophages in PDAC is associated with increased tumour size, earlier lymphatic metastasis, earlier post-surgical local recurrence and ultimately a poorer prognosis[65,66]. Partecke et al show that culture media from pancreatic cancer cells growing *in-vitro* can polarise macrophages to the M2 phenotype and that culture supernatants from M2 macrophages induce a significant increase in pancreatic cancer cell growth[64]. There is also evidence that other cells in the TME may be involved in macrophage polarisation. In cancer, T-cell derived IL4 in addition to tumour-cell derived colony-stimulating factor-1 (CSF-1) has been shown to elicit a switch from the M1 to the M2 phenotype as part of an immune evasion strategy[67]. It is therefore becoming increasingly evident that TAMs promote PDAC tumourogenesis and growth through a variety of mechanism and are thus a critical component of the PDAC TME.

1.1.3.1.2 Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are a commonly expanded cell population in a variety of tumour types, including PDAC[47]. They originate from bone marrow myeloid progenitor cells and are undifferentiated, immature cells of the

myeloid lineage that include immature stages of macrophages, granulocytes and dendritic cells (DCs)[60,68,69]. Like macrophages, they show phenotypic plasticity. This phenotypic regulation is dependent on signals received from cancer cells and the tumour stroma[70]. There are two main phenotypes: polymorphonuclear MDSCs, sometimes known as granulocytic MDSCs (G-MDSCs), and monocytic MDSCs (M-MDSCs)[70,71]. In mice these can be identified as $CD11b^{+}Ly6G^{+}Ly6C^{lo}$, and $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ respectively[71]. Differentiating G-MDSCs from subpopulations of neutrophils is contentious and currently lacks clear definition. Therefore for simplicity, this group will be referred to as G-MDSCs hereafter[72].

In humans, MDSCs are not only increased in the pancreatic TME but also in the circulation, which correlates with clinical stage and circulating Treg levels[73,74]. In addition to circulating in the blood, MDSCs are also frequently detected in the bone marrow and spleen of pancreatic cancer patients, raising the possibility that they are expanded systemically before being recruited to the tumour[47].

There is growing evidence that after MDSCs are recruited and activated by tumour-cell and stromal derived signals, they then in turn directly support tumour progression, neovascularization, metastasis, and are a major source of immunosuppression within the TME. Genetic deletion or inhibition of CXCR2, a receptor shown to be up-regulated on neutrophils/MDSCs in human pancreatic cancer have been shown to reduce metastasis in *in-vivo* models[75]. Studies utilising the KPC mouse model of pancreatic cancer have shown that cancer cell derived GM-CSF is a key cytokine in the development and recruitment of MDSCs that promote pancreatic tumour growth by suppression of cytotoxic $CD8^{+}$ T-cell activation[50,76]. Additionally, treatment of mice with GM-CSF depleting antibody to selectively inhibit GM-CSF, reduced pancreatic tumour development[76].

MDSCs inhibit cytotoxic T-cell activity to support tumour growth in a number of ways[60]: 1) they produce large quantities of arginase and sequester cysteine, thereby reducing availability of L-arginine and cysteine to T-cells within the TME, thus hampering T-cell protein synthesis and proliferation, 2) production of reactive oxygen species (ROS) leading to apoptosis of T-cells and NK cells, 3) production of immunosuppressive cytokines including IL10 and TGF-β which inhibits $CD8^{+}$ T-cell

function, 4) down-regulation of L-selectin on $CD4^+$ and $CD8^+$ T-cells thus reducing T-cell homing and 5) up-regulation of immune checkpoint PD-L1 which binds to T-cell PD-1 receptor to down regulate T-cell activity. Notably, MDSCs isolated from pancreatic cancer patients were found to express high levels of PD-L1 causing significant inhibition of T-cell proliferation *in-vitro*[77]. There is therefore a wealth of evidence confirming MDSCs as major contributors to the immunosuppressive pancreatic TME.

1.1.3.1.3 Tumour infiltrating lymphocytes

Tumour infiltrating lymphocytes (TILs) are critical components of the TME in cancer and have a variety of contrasting functions. TILs form part of the adaptive immune response, becoming activated after antigen exposure. There are two main TIL classes: cytotoxic $CD8^+$ T-cells forming part of the tumour-specific cellular adaptive immunity and $CD4^+$ T-helper (T_H) cells.

Activated $CD8^+$ T-cells are the main cytotoxic effector cell type within the TME, attacking tumour cells that present tumour-associated peptides via MHC class I on their surface. $CD8^+$ T-cells can directly eliminate tumour cells via secretion of effector cytokines IFN-γ and tumour necrosis factor (TNF)-α, localised release of cytotoxins such as perforin, and granzyme B, and indirectly by induction of macrophage tumouricidal activity[78]. However in PDAC, cancer cells frequently down-regulate or lose MHC class I expression thereby preventing attack from $CD8^+$ T-cells[79] and also express PD-L1[80] inducing T-cell anergy or death via PD-1. Additionally TGF-β derived from pancreatic cancer cells and other sources within the TME inhibits $CD8^+$ T-cells from expressing effector cytotoxins[60,79]. $CD8^+$ T-cells heavily infiltrate early PanINs but their numbers diminish through progression to carcinoma[81]. $CD8^+$ T-cell infiltration is very poor in PDAC and correlates with prognosis[48,82,83]. Activated pancreatic stellate cells (PSCs) have been shown to produce the chemokine CXCL (C-X-C motif ligand) 2, mediating $CD8^+$ T-cell sequestration, thus preventing $CD8^+$ T-cell contact with malignant epithelial cells in the juxtatumoral compartment[84]. Other immunosuppressive cell types such as MDSCs, Tregs and certain $CD4^+$ T-helper cell subsets also suppress cytotoxic $CD8^+$ T-cells through various effector cytokine production[47].

$CD4^+$ T-helper cells can be subdivided based on the cytokine profile they secrete[85,86]: T_H1 cells producing IFN-γ, T_H2 cells producing IL4, IL5 and IL13, T_H17 cells producing IL17, and immunosuppressive Tregs secreting TGF-β[87]. There are also more recently described subsets of $CD4^+$ T helper cells such as T_H9, T_H22, and T-follicular helper (T_{FH}) cells[88]. $CD4^+$ T-cell differentiation is determined by exposure to particular chemokines and cytokines they encounter in the TME. IFN-γ, IL12 mediate T_H1 differentiation through activation of transcription factor T-Bet[89], STAT (signal transducer and activator of transcription)1 and STAT4[90]. In most human cancer types including pancreatic cancer[47], T_H1 cells augment the anti-tumour immune response through enhancing M1 macrophage activity, $CD8^+$ T-cell mediated killing and through recruitment of inflammatory cells to the tumour site[91]. IL4, IL2 and IL33 induce T_H2 differentiation through activation of GATA-3. IL6 is also thought to determine T_H1/ T_H2 fate through induction of Nuclear Factor of Activated T-cells (NFAT)c2 expression and STAT3 dependent up-regulation[92,93] of the specific T_H2 transcription factor c-maf (V-maf musculoaponeurotic fibrosarcoma oncogene homolog)[92,94]. A second mechanism of IL6 regulatory control of the T_H1/ T_H2 balance towards T_H2 is mediated through up-regulation of Silencer of Cytokine Signalling (SOCS)1 expression, leading to reduced IFNγ expression and loss of positive feedback through IFNγ signalling[95]. As a consequence, $CD4^+$ T-cells become unresponsive to IFNγ signalling, and display reduced T_H1 differentiation. More recent evidence has shown that IL6 inhibition in various murine cancer models increases T_H1 differentiation[92,96–98]. Specifically in a murine model of pancreatic cancer, IL6 inhibition by use of a neutralising antibody, in addition to PD-L1 inhibition, led to an increase in T_H1 differentiation, reduced tumour progression and increased infiltration of effector $CD8^+$ T-cells into the tumour[98]. Thus a potential mechanism for impaired T_H1 responses and an increased immunosuppressive T_H2 response in tumour bearing hosts may be linked to an increase in IL6. The pancreatic TME has been reported to have a higher frequency of T_H2 cells when compared to T_H1 and this infiltration correlates with reduced survival in pancreatic cancer patients[99]. Thus, the GATA3:Tbet ratio in post-surgical samples has been suggested as an independent predictive survival marker in PDAC patients[47]. T_H2 cells facilitate pancreatic tumour growth through various mechanisms. For example, secretion of

IL13 and IL5 by T_H2 cells is thought to drive collagen synthesis and deposition[100], contributing to the highly fibrotic stroma observed in PDAC. Importantly, cytokines released by T_H2 cells induce M2 macrophage polarisation, further inducing an immunosuppressive, pro-tumour TME[47].

T_H17 cells are another T-helper subset found in the pancreatic TME. IL6 and TGF-β are known as the critical cytokines in RORγt induction in naive $CD4^+$ T-cells, in turn driving differentiation to the T_H17 phenotype. Maintenance of this phenotype also requires IL21 and IL23[101]. The main effector cytokine that T_H17 cells secrete is IL17, but they can also secrete IL21 IL22, and CCL20[102]. T_H17 cells have been identified in a number of human cancers including pancreatic, ovarian[103], gastric[104], renal cell carcinoma[105] and prostate cancers[106]. The role of these cells in cancer is controversial, having found to be both anti-[107–110] or pro-tumourigenic[111] in different contexts[102]. Like many immune cell subsets, T_H17 cells exhibit a certain degree of plasticity; exposure to IL12 induces differentiation into IFNγ producing T_H1 cells, augmenting the action of $CD8^+$ T-cells[102]. In contrast, cancer cell derived TGF-β can induce T_H17 cells to become regulatory and promote host tolerance[112]. In mouse models of pancreatic cancer, T_H17 cells have been shown to both promote[113] and inhibit[114] pancreatic cancer progression. However in human pancreatic cancer, T_H17 cells and IL17 secretion is associated with increased clinical stage and a poorer prognosis[108,115,116].

Regulatory T-cells (Tregs) are highly immunosuppressive cells characterised by the expression of the transcription factor forkhead box P3 ($FoxP3^+$) and secretion of IL10 and TGF-β. Physiologically Tregs suppress unwanted autoimmune reactions, a function exploited by cancer cells. In addition to driving expression of immunosuppressive cytokines, FoxP3 also suppresses expression of the inflammatory cytokines TNF-α, IFNγ, IL17 and IL4, and promotes expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), indoleamine 2,3-dioxygenase (IDO), CD39 and CD73[117,118]. Expression of CTLA-4 by Tregs results in the down-regulation of CD80 and CD86 expression on DCs through trans-endocytosis and subsequent degradation, thereby depleting the co-stimulatory signal required for T-cell activation[119]. IDO is a protein that functions as part of the

kynurenine pathway that catabolizes tryptophan to kynurenines, leading to a reduction in the availability of this essential amino acid for effector T-cells including cytotoxic T-cells, helper T-cells, and natural killer (NK) cells, resulting in arrest of their cell cycle and apoptosis[118,120–122]. Ectoenzymes CD39 and CD73 expressed on the cell surface hydrolyse extracellular ATP and ADP to adenosine. Reduced environmental availability results in local inhibition of effector T-cell proliferation and reduced DC function[123,124]. Mechanisms such as these result in repression of M2 TAMs, NK cells, DCs and tumour-specific $CD4^+$ and $CD8^+$ T-cells to promote tumour progression[60]. Tregs numbers are increased in PDAC compared with normal pancreatic tissue and this increase is a negative prognostic factor[48]. They can be identified from early PanIN lesions and infiltration increases through carcinogenesis whilst inversely correlating with $CD8^+$ T-cell infiltration[81,125,126].

1.1.3.1.4 Dendritic cells

Dendritic cells (DCs) are 'professional' antigen presenting cells (APCs) that are essential for the adaptive immune response[127]. They prime an adaptive immune response by engulfing tumour material, proteolytically degrading proteins to generate peptide antigens, and presenting these peptide antigens to $CD4^+$ and $CD8^+$ T-cells using both MHC class I and II molecules respectively. DCs can also activate NK cells leading to potent cytotoxic responses in cancer[128]. DCs are derived from the bone marrow and can exist in mature and immature forms within the TME[127,129]. In PDAC, the presence of increased circulating DCs and DCs within the tumour microenvironment are associated with prolonged survival. However DCs infiltrate the PDAC TME in low numbers and often exhibit maturation defects or impaired function[130–132]. It is thought that cancer cell derived IL6, vascular endothelial growth factor (VEGF), macrophage colony stimulating factor (M-CSF) and also the hypoxic cancer TME leads to this impaired maturation and function[47]. More recently immunosuppressive DC subsets supporting Tregs and suppressing $CD8^+$ T-cells have been identified in PDAC[133].

1.1.3.1.5 Natural killer cells

Natural killer (NK) cells form part of the first line, innate immune response[134], exerting their toxic effects through release of perforin and granzyme-mediated effects and through activation of the caspase pathway[135]. Increased circulating NK

cells are associated with a better prognosis in pancreatic cancer patients[136]. However typically low numbers of activated NK cells are found within the PDAC TME with reduced expression of activating receptors such as NKG2D (natural-killer group 2, member D) receptor[79,137].

1.1.3.2 The fibrotic TME in PDAC

The desmoplastic reaction surrounding pancreatic cancer cells is extensive, accounting for a large proportion of the tumour mass. In addition to the infiltrating immune cells, this desmoplastic stroma is composed of blood vessels and large amounts of extra-cellular matrix (ECM), principally laid down by cancer-associated fibroblasts (CAFs)[138]. As with the immune cell compartment of the PDAC TME, there is a large amount of cross talk between cancer cells and the cellular and non-cellular components of this desmoplastic stroma. Through this cross talk, cancer cells enable expansion of the desmoplastic reaction, which in turn supports and promotes cancer cell proliferation and tumour progression.

1.1.3.2.1 Cancer associated fibroblasts

Cancer-associated fibroblasts (CAFs) are the main effector cells establishing the desmoplastic reaction[138]. Pancreatic stellate cells (PaSCs) are the most important source of CAFs, the other being recruited from bone marrow stem cells[139]. Quiescent PaSCs are present in the normal pancreas in low numbers and contain large numbers of cytoplasmic lipid droplets that are high in vitamin A; these are lost once PaSCs become activated under inflammatory conditions. Other morphological changes occur upon activation such as taking on an α-smooth muscle actin (α-SMA) expressing myofibroblast phenotype, in addition to loss of fat droplets and increased proliferation[39,140]. CAFs derived from these PaSCs play a central role in orchestrating the fibrotic reaction in PDAC. They are responsible for laying down large amounts of ECM proteins including collagen I, III, XI, and fibronectin, and can remodel the ECM through expressing matrix metalloproteases (MMPs). Furthermore, they also produce multiple growth factors and cytokines such as platelet derived growth factor (PDGF), fibroblast growth factor: (FGF), TGFβ, IL-1β, IL8, VEGF and CXCL12[43,141–143], all which have shown to promote cancer progression and/or promote the desmoplastic reaction. Therefore, CAFs play a central role in

orchestrating the characteristic desmoplastic reaction that is a feature of PDAC, and contributes to immune suppression within the TME. The relationship between CAFs and pancreatic cancer cells is further supported by *in-vitro* studies utilising co-culture methods demonstrating cross talk between CAFs and pancreatic tumour cells, both enhancing the proliferation of the other[44,144,145]. However, there is controversy in the literature regarding the role of this fibrotic reaction *in-vivo*. Several studies have shown that the stromal reaction creates a barrier to delivery of chemotherapeutics, thus provides protection from cancer treatment strategies such as chemotherapy, immunotherapy and also radiotherapy[146]. On the other hand, several mouse model studies have shown that depletion of this fibrous reaction alone leads to a more aggressive cancer phenotype[147,148]. Through production of VEGF in response to hypoxia and inflammation, CAFs are also intimately linked to the angiogenic response in PDAC, which will be discussed in more detail in the next section.

1.1.3.2.2 Endothelial cells

In many cancers, including PDAC, the supply of oxygen, nutrients and growth factors increases as the tumour progresses. Angiogenesis is the creation of new blood vessels and vascular networks from pre-existing vasculature, enabling increased supply of these resources to enable accelerated tumour growth[149]. This process requires molecular communication between the cancer cells and surrounding stroma via chemokines and cytokines to facilitate this process. The key mediator of this process is VEGF, a growth factor produced by pancreatic cancer cells, TAMs, CAFs and endothelial cells, levels of which are a negative prognostic marker in human PDAC[147]. Angiogenesis can be driven by hypoxia; direct intra-tumoral oxygenation measurements have revealed that the PDAC tumours are significantly hypoxic[150]. Under hypoxic conditions CAFs up-regulate hypoxia-inducible factors[151,152], collagen I and VEGF[153,154]. However the resultant blood vessels are often morphologically abnormal and characterised by disordered branching and increased permeability, leading to poor perfusion and further contributing to the hypoxic environment[155]. This is evident in PDAC as there exists a poorly functional vascular supply within the tumour[45] contributing to therapy resistance by reducing delivery of cytotoxic agents and through selecting for slow-cycling, highly drug-resistant cancer cells[39].

1.1.3.3 Cytokines in the PDAC TME

Pancreatic cancer cells orchestrate the composition of their surrounding TME to promote tumour progression and to maintain an immunosuppressive environment in order to protect themselves from immune-mediated destruction. Communication between the neoplastic epithelial cells and the cells comprising the tumour stroma is mediated through autocrine and paracrine signalling pathways involving a wide variety of cytokines and chemokines to modulate the TME and promote pancreatic tumour progression[156]. In addition to cytokines, cancer cells communicate to the surrounding stroma via direct interactions between cancer cells and ECM components via cell surface receptors such as integrins signalling via focal adhesion complexes[157]. Multiple studies exist implicating cytokines and chemokines in cancer and a variety of other pathologies. In PDAC, increased circulating levels of several cytokines, including IL6, IL1-β, IL10 and TGF-β have been shown to promote tumour progression by modulating the TME, and higher circulating levels of these are generally associated with poorer clinical outcomes[156,158]. The primary tumour site is thought to be the source of elevated cytokine production; Bellone et al. observed m-RNA over-expression of several cytokines, such as IL6, IL8, IL-1β, IL10, and TGF-β from patient primary tumour site which correlated with higher circulating levels and reduced patient survival[159]. I will now discuss the role of some of these cytokines in PDAC in more detail.

1.1.3.3.1 Interleukin-6 (IL6)

Interleukin-6 (IL6) is an important pro-inflammatory cytokine, mediating inflammatory responses to infection and injury whilst also regulating cell proliferation and differentiation. Increased levels of IL6 are associated with several auto-immune diseases[160] and various cancers[161] via regulation of immune cell balance. High levels of IL6 together with TGF-β induces the development of Th17 cells from naive $CD4^+$ T-cells, and inhibits TGF-β induced differentiation[162]. Additionally it can regulate the T_H1/T_H2 balance in favour of T_H2 polarisation[92–94,96–98].

Increased IL6 levels have been reported in a number of cancers, including breast[163], prostate[164], endometrial[165], renal cell carcinoma[166], oral squamous cell carcinoma (SCC)[167], multiple myeloma[168], colorectal cancer[169] and pancreatic cancer[170]. Plasma

levels of IL6 have a highly inverse relationship with patient survival in patients with metastatic or untreatable PDAC[171].

IL6 engagement with the membrane bound IL6 receptor (IL6Rα) or the soluble form of the IL6R (sIL6R) leads to engagement of the common signal transducing receptor chain GP130 (glycoprotein 130)[172], initiating downstream activation of JAK/STAT (Janus kinases/ Signal transducer and Activator of Transcription proteins), MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase) pathways[173,174]. These pathways are involved in cell proliferation, survival and immune evasion in cancer. For example, IL6 induced STAT3 signalling directs myeloid cells to express immune-suppressive molecules such as arginase and VEGF[175,176] whilst also being associated with increased differentiation of macrophages to the pro-tumour M2 macrophage phenotype[176]. Additionally IL6 can decrease DC MHC-II, CD80/86 and IL12 expression[177] and can re-program DC differentiation to regulatory IL10 producing DCs[178]. This therefore reduces the DCs ability to activate T_H1 cells and cytotoxic T-cells, thus further contributing to the immunosuppressive TME. IL6 induced STAT3 activation is relevant to clinical outcome in PDAC; activation of the IL6Rα-JAK-STAT3 pathway in surgically resected PDAC is associated with reduced survival[179] and studies have shown STAT3 is critical in the early initiation[180] and progression[93,181] in KRAS driven murine models of PDAC. Additionally, when the aggressive KRAS driven PDAC murine model iKras*, created using three genetically modified mouse strains to generate triple transgenic p48-Cre;R26-rtTa-IRES-EGFP;TetO-$Kras^{G12D}$ mice, were crossed with IL6 -/- mice, PDAC progression is completely arrested[182].

There are a variety of cellular sources of IL6 in the PDAC TME including macrophages[93], fibroblasts and epithelial cells[182] and malignant epithelial cells such as PDAC cells, which are thought to be a major source of IL6 within the TME. IL6 is secreted by a number of PDAC cell lines[170] and PDAC cancer cell derived IL6 has been shown to activate STAT3 in an autocrine fashion[181]. IL6 specifically secreted by cancer cells has also been shown to alter the TIL balance *in-vivo* with subsequent influence on tumour growth[114,183]. Additionally, Bellone el al demonstrated that pancreatic cancer cell derived IL6 acted together with IL10 and TGF-β to reduce activation and proliferation of dendritic cells (DCs), resulting in a reduced anti-

tumour response[184]. Conversely however, Gnerlich et al. observed overexpression of IL6 in cancer cells derived from a TGF-β secreting murine model of pancreatic cancer skewed the balance from a Treg to a T_H17 predominant environment, with a resultant delay in tumour growth and improved survival times. Therefore, though some controversy exists regarding the exact role of IL6 in PDAC, cancer cell derived IL6 is considered to play a crucial role.

The growing body of evidence identifying IL6 as an important player in PDAC progression has ignited interest in the development of drugs targeting either IL6 itself or the IL6Rα-JAK-STAT3 pathway. It is thought that inhibiting IL6 will reprogram the TME to render it more responsive to immunotherapy regimens. In support of this hypothesis, a phase II clinical trial of the JAK 2 inhibitor ruxolitinib reported improved survival in pancreatic cancer patients with metastatic disease[185]. A phase II clinical trial is also underway in the USA aimed at evaluating the anti-tumour efficacy of the IL6 receptor antibody inhibitor tocilizumab in patients with unresectable pancreatic cancer (clinical trials.gov NCT02767557), however, the outcome has not been reported at this time.

1.1.3.3.2 Interleukin-1-beta (IL1-β)

Interleukin-1-beta (IL1-β) is another pro-inflammatory cytokine that like IL6, plays a role in both inflammatory disease[186] and is implicated in the progression of pancreatic cancer. IL1-β has a reported role in regulating PDAC invasion and metastasis[187,188]. Inhibition of IL1-β results in a reduction in pancreatic tumour size and tumour related inflammation[189]. Acting primarily through nuclear factor κB (NF-κB), IL1-β can also contribute to chemoresistance in pancreatic cancer[190]. Therefore, IL1-β can impact PDAC progression in multiple ways.

1.1.3.3.3 Interleukin-10 (IL10)

Interleukin-10 (IL10) is an anti-inflammatory cytokine. Acting through JAK-STAT signalling pathways, this cytokine prevents excessive tissue damage during infection[191]. Several studies have identified high levels of IL10 expression in PDAC, and levels correlate with worse overall survival and increasing tumour stage[159,192]. It is thought that IL10 can influence the inflammatory cell population within the TME. For example, IL10 has been found to reduce activation of natural killer (NK) cells and DCs[184,193], and it can shift T-cell cytokine production from that of a T_H1

phenotype to a more pro-tumour T_H2 type phenotype[184].

1.1.3.3.4 CC chemokines

Both CCL5/ Regulated Upon Activation Normal T-Cell Expressed and Presumed Secreted (RANTES)[194] and CCL2/MCP-1, (monocyte chemoattractant protein-1)[195] have been found to be important in PDAC. CCL5 is involved in PDAC immune evasion as it has been shown to recruit immunosuppressive CCR5 positive Tregs into the TME[196]. In a murine model of SCC FAK dependent regulation of CCL5 expression in cancer cells led to elevated levels of Tregs in the TME and subsequent suppression of the anti-tumour $CD8^+$ T-cell response[197].

1.1.3.3.5 CXC chemokines

CXCR2/ interleukin-8 (IL8) is overexpressed in PDAC[159] and negatively correlates with prognosis[198]. In PDAC, its main role is in angiogenesis through induction of VEGF[199]. However, it has also been shown that tumour cell derived CXCR2 can act in conjunction with fibroblast derived chemokine CXCL12 within the pancreatic TME to stimulate proliferation, migration and invasion of cancer cells[200]. Recently Jiang et al identified that levels of pancreatic cancer cell derived CXCL12 is modulated by FAK, and that this chemokine induced the proliferation of pancreatic fibroblasts cultured *in-vitro*, implying that CXCL12 may drive stromal fibroblast expansion in the pancreatic TME leading to excessive collagen deposition[142].

1.2 Immunotherapy

The immune system is fundamental to the body's defence against infectious disease and also elimination of cancerous cells. In recent decades, our understanding of the immune system and its intimate relationship with cancer has evolved; the interaction between immune cells and cancer cells results in either effective immunosurveillance or alternatively tumour growth and progression[79]. This knowledge has led to the development of cancer immunotherapy. The aim of cancer immunotherapy is to harness the power of the immune system, enabling recognition and eradication of tumour cells and generation of a long-lasting protective response[201,202], whilst sparing normal healthy tissue.

Despite the remarkable success of cancer immunotherapy in the treatment of cancers including melanoma[203] and non-small-cell-lung cancer[204,205], no overall survival benefit has yet been demonstrated in clinical trials using single agent immunotherapy in PDAC patients[206,207]. However, immunotherapy is still regarded as an emerging therapeutic option in PDAC. As described in previous sections of this thesis, the PDAC TME is regarded as highly immunosuppressive. This represents both a significant obstacle to the success of immunotherapy, but also a potential therapeutic opportunity. An improved understanding of this uniquely fibrotic and immunosuppressive TME may have an important role to play in the future treatment of PDAC patients, by uncovering novel targets resulting in new approaches to cancer immunotherapy, or selection of more effective drug combinations targeting different aspects of the immunosuppressive TME[208]. A recent study by Jiang et al revealed encouraging evidence suggesting that immunotherapy could be effective in PDAC with optimal combinations of therapies. They demonstrate that FAK inhibition can result in broad regulation of the fibrotic and immuno-suppressive PDAC TME, rendering pancreatic tumours responsive to a combination of gemcitabine, one of the current chemotherapies used in the treatment of PDAC, and anti-PD-1 plus anti-CTLA-4 immune checkpoint blockade[142]. These results have seen rapid clinical translation and a Phase I clinical trial testing FAK inhibition in combination with gemcitabine and anti-PD-1 in patients with pancreatic cancer is currently underway in the USA (clinicalTrials.gov NCT02546531). A further clinical trial testing the safety, tolerability, and anti-tumour efficacy of FAK inhibition in combination with anti-PD1 in patients with pancreatic cancer, mesothelioma, and non-small-cell lung cancer is also underway in the UK (ClinicalTrials.gov, NCT02758587).

1.2.1 Mechanisms of escape

Preclinical data suggests immune evasion is the basis of the underwhelming response to immunotherapies thus far in the treatment of pancreatic cancer[40]. Immunotherapy may only elicit an effective treatment response when multiple facets of the protective armoury shielding PDAC against immune destruction and promoting its development are targeted concurrently. There are several factors contributing to pancreatic cancer's ability to escape immune recognition and circumvent the actions of single agent immunotherapies. First, as described earlier in this thesis, the PDAC TME is

highly infiltrated with immunosuppressive cell types secreting immunosuppressive cytokines blocking the function of $CD8^+$ T-cell tumour recognition and clearance and creating a site of immune privilege. Second, the intense desmoplastic and fibrotic environment not only acts as a physical barrier to infiltration of immune cells and immunotherapeutic agents as well as other cytotoxic agents, but is also directly immunosuppressive[39,44,144,145]. Thirdly, analysis of mutational signatures have revealed that most pancreatic cancers are poorly immunogenic or display high tumour heterogeneity[32,35,40] and therefore the less immunogenic clones could potentially be selected for in response to immunotherapy. Furthermore, neoantigen quality has been found to decline on cancer progression[209] resulting in pancreatic cancer becoming less visible to the immune system as the tumour develops. This may in part be due to the genetic instability of pancreatic cancer, with consequential loss of tumour antigen expression and survival of cells expressing tumour antigen in a process known as 'immunoediting'[210]. Finally, pancreatic cancer cells prevent immune recognition by altering expression of molecules on their cell surface; either by overexpressing inhibitory signals such as immune checkpoint ligands or down regulating antigen presenting pathways resulting in reduced expression of major histocompatibility complex (MHC) I proteins, TAP (transporter associated with antigen processing) proteins and LMPs (latent membrane proteins)[211].

1.2.2 Cancer vaccines

A number of synthetic and cellular-based cancer vaccines have been trialled in the treatment of pancreatic cancer[137]. Cancer vaccines aim to break the acquired immune tolerance to cancer cells by expansion and activation of the DC population, resulting in generation of an anti-cancer humoral and/or cellular immune response[67]. Synthetic vaccines are generally comprised of proteins or peptides corresponding to a pre-determined antigen that induces a T-cell response. Clinical trials have been conducted targeting VEGF[212–214], MUC-1 (Mucin 1)[215,216], survivin[217], telomerase[218,219], and KRAS peptides[220–223]. Some promising results were found at 10-year follow up subsequent to anti-KRAS peptide vaccination[220], but in general, use of synthetic vaccines have not translated into substantial improvement in patient survival. More favourable responses have been found using cellular based vaccines. The antigen source is generally whole cells and therefore cellular based vaccines

have multiple epitopes[224]. This potentially results in a more robust immune response. For example, GVAX (a GM-CSF vaccine) improved overall patient survival in phase II studies when used in combination with low dose cyclophosphamide to deplete Tregs[225], 5-FU (fluorouracil) based chemo-radiation[226], or with a live-attenuated strain of Listeria monocytogenes–encoded mesothelin (CRS-207)[227]. Additionally, phase II studies with Algenpantucel-L, a whole cell vaccine manufactured from irradiated allogeneic pancreatic cancer cells expressing the murine α-GT enzyme, demonstrated positive results in those patients showing an antibody response to the vaccine[228,229]. Another promising vaccine approach is dendritic cell vaccines. This involves isolating DCs from the patient's peripheral blood mononuclear cells (PBMCs), stimulating them *ex-vivo* and re-injecting them back into the patient. This approach has shown significant increase in patient survival in prostate cancer and subsequently these has been approved for use in cancers such as prostatic cancer[230]. DC vaccines have also recently been shown to increase overall survival and disease free interval in pancreatic cancer patients[231]. Adoptive cell transfer (ACT) is another promising immunotherapy treatment in PDAC. This involves the infusion of allogenic tumour reactive T-cells or autologous T-cells derived from patient PBMCs, tumour draining lymph nodes or tumour tissue, which are isolated and expanded *ex-vivo*[232]. The most widely used ACT approach is with T-cells genetically modified to express chimeric antigen receptors (CARs) specific to tumour-associated antigens. Despite showing great promise in pre-clinical studies[233–236], CAR T-cell therapy has yet to demonstrate significant advantages in the clinical setting. It is thought that the immunosuppressive TME in PDAC is the source of failure in objective responses in the clinic[237] and strategies are underway combining CAR T-cell therapy with methods to impede TME immunosuppression[40].

1.2.3 Macrophage targeted immunotherapy

The pro-tumoural functions of TAMs have put the spotlight on macrophage-targeted therapies as a potential treatment in pancreatic cancer, either through ablation or re-polarisation of macrophages within the tumour microenvironment. Gemcitabine in combination with an agonist of macrophage surface marker CD40, has been shown to elicit tumour regression through promotion of macrophages with an anti-tumour phenotype[61,238]. Another strategy for targeting TAMs is to block monocyte

recruitment into tumour tissues by blocking CCL2. Use of the CCL2 antagonist PF-04136309 in a murine model of PDAC led to TAM depletion, subsequent reduction in tumour growth and inhibition of distant metastasis[239]. Additionally, objective tumour responses were seen in 49% of patients with inhibition of CCR2 in combination with FOLFIRINOX in a phase Ib clinical trial (NCT01413022)[240]. Colony-stimulating factor 1 receptor (CFS1R), expressed by macrophages and monocytes, has also shown potential in pre-clinical studies. Inhibition of this receptor has been shown to amplify antigen presentation and T-cell immune responses against murine pancreatic cancer[241]. Another promising immunotherapy targeting macrophages and enhancing their anti-tumour activity is Decoy receptor 3 (DcR3). DcR3 is a member of the TNF receptor superfamily and found to down regulate antigen presenting genes such as MHC-II and HLA-Dr (Human Leukocyte Antigen – antigen D Related)[242]. DcR3 is up-regulated in pancreatic cancer[243] and correlates with reduced survival[60].

1.2.4 Immune checkpoint inhibition

The immune system has a number of intrinsic mechanisms known as immune checkpoints that maintain self-tolerance to prevent autoimmunity and avert overwhelming immune reactions during infection[244]. Cancer cells have developed various strategies to commandeer some of these mechanisms to achieve cancer cell self-perseverance by inhibiting both the innate and adaptive immune system, leading to TME immunosuppression[245]. This has lead to treatment strategies targeting these co-stimulatory receptors and ligands that block the inhibitory receptor-ligand interaction on T-cells, cancer cells and other immune cells. Thus far the main focus has been on cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) and programmed death protein-1 (PD-1)[79], with other potential targets such as lymphocyte activation gene-3 (LAG-3)[246] and T-cell immunoglobulin and mucin domain-3 (TIM-3)[247] currently under investigation.

CTLA-4 is an inhibitory receptor expressed on T-cells, regulating early stages of T-cell activation. Upon CD80 or CD86 ligand binding, T-cell activation is prevented[248]. Anti CTLA-4 monoclonal antibodies (ipilimumab, tremelimumab) have been employed successfully in clinical trials against metastatic melanoma and are now approved for clinical use. Metastatic melanoma patients treated with ipilimumab, in

combination with the alkylating agent dacarbazine, have an improved overall survival of 3.7 months, compared to dacarbazine alone[249]. Clinical trials with CTLA-4 inhibition are currently underway in pancreatic cancer (NCT00556023, NCT01473940) but despite the success in melanoma, no improvement in survival has yet been reported when used as a single agent in PDAC[206], suggesting that combination therapy to target other areas of cancer immune evasion may be required.

The PD-1 receptor is another immunotherapy target that has shown significant promise. Unlike CTLA-4, PD-1 expression is not limited to T-cells and can be expressed on a number of immune cell populations including B-cells and NK cells. Upon binding to either of it's two ligands: PD-L1(also known as B7-H1/ CD274) and PD-L2 (also known as B7-DC/ CD273), PD-1 functions to inhibit cytotoxic T-cell activation[250] and enhance Treg activation and proliferation[251] during an inflammatory response in order to prevent autoimmunity. Both PD-L1 and PD-L2 are expressed on antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs). Whilst PD-L2 expression is generally thought to be restricted to these cells, PD-L1 is also expressed on a variety of other cell types such haematopoietic cells and non-lymphoid cells including parenchymal cells and vascular endothelial cells in the peripheral tissues[252]. Ligation of PD-1 on T-cells leads to SHP-2 recruitment and subsequent de-phosphorylation and inactivation of Zap 70, a major integrator of T-cell receptor (TCR)-mediated signalling, resulting in reduced effector function, proliferation and survival of $CD8^+$ T-cells[253]. Additionally, PD-1 is expressed on Tregs and activation is thought to enhance Treg activation and proliferation[254], again dampening the immune response. Physiologically the PD-1 receptor plays an inhibitory role in regulating T-cell activation in peripheral tissues to restrain exaggerated T-cell responses to infection and to prevent autoimmune reactions against self antigens[250]. It is now known that cancer cells are able to hijack this mechanism through over-expression of PD-L1. Cancer cell PD-L1 binding of T-cell PD-1 leads to evasion of the anti-tumour response via effector T-cell deactivation and Treg activation, thus PD-1 has recently become an attractive therapeutic target[253]. Considerable research has gone into PD-L1 expression in various cancer types but less emphasis has gone into PD-L2, the other PD-1 ligand. Response rates in clinical trials to PD-1 or PD-L1 inhibitors were 17–38% in advanced melanoma and 10–18% in advanced non-small-cell lung cancer[255,256]. PD-L1 expression has been found to negatively correlate with pancreatic cancer patient

survival and a survival benefit has been demonstrated with use of PD-L1 blockade in PDAC murine models[257]. Both PD-L1 and PD-L2 have been found to be over expressed in post surgical PDAC specimens[258]. Despite this, no objective responses have yet been found in clinical trials in pancreatic caner when anti-PD-L1 antibodies were used alone[80,259]. Clinical trials with combination therapy are currently underway and one phase 1b trial with ipilimumab in combination with GVAX demonstrated increased survival in metastatic pancreatic cancer patients when compared to ipilimumab alone[260]. Combinational therapies targeting different arms of the immunosuppressive TME in order to overcome other components of the immunosuppressive TME that mediate immune evasion may therefore be more effective in PDAC treatment. Jiang et al reports a recent example of pre-clinical success using combinational therapy. In this study they utilised mouse models of pancreatic cancer, previously found to be unresponsive to inhibition of immune checkpoint blocker PD-1 alone, and found that the addition of a FAK inhibitor (VS-4718) to a combination of anti-PD-1 and gemcitabine, resulted in increased overall mouse survival, when compared to the combination of anti-PD1 and gemcitabine[142].

1.3 Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor tyrosine kinase[261], originally identified in 1992 as a 125kDa protein that was tyrosine phosphorylated in Rous sarcoma virus-transformed chick embryos and localized to regions on the cell surface termed focal adhesions (FAs)[262]. Further characterisation has revealed FAK as a critical signal integrator, acting downstream of integrin and growth factor receptors, scaffolding macromolecular complexes to direct signals from the extracellular environment into the cytoplasmic space. This co-ordinates an array of cellular processes, many of which are important in cancer including: cell cycle progression, adhesion, polarisation, migration, apoptosis, proliferation and survival[263]. Multiple studies have revealed elevated FAK expression in a range of invasive cancers[264] and this elevation generally correlates with poorer survival rates[265], highlighting the potential for FAK as a therapeutic target in cancer.

1.3.1 Structure and function of FAK

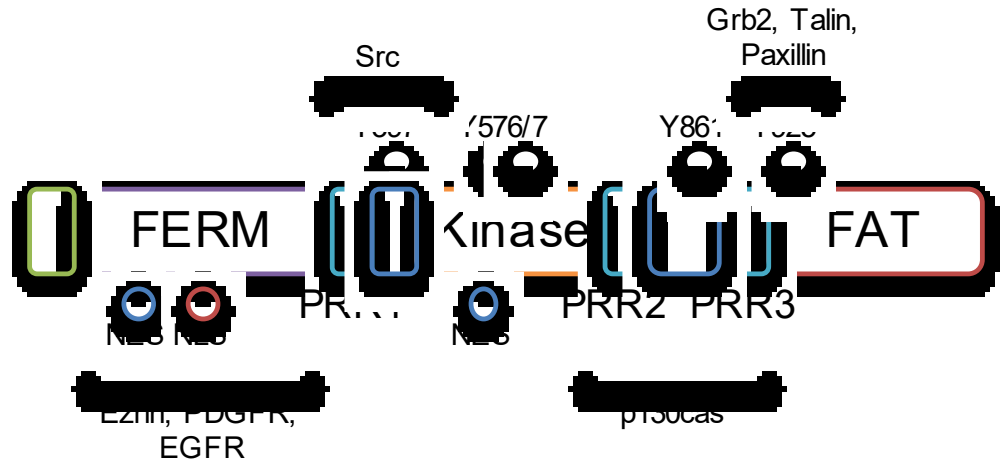
The FAK protein is highly conserved between species with approximately 90% amino acid sequence homology between human and mouse[266]. FAK consists of 3

functional domains: an N-terminal FERM (Four-point-one, Ezrin, Radixin and Moesin) domain, a central kinase domain, and a C-terminal FAT (Focal Adhesion Targeting) domain[267] (**figure 1.3**). Studies have revealed that deletion of the FERM domain increases FAK activity[268]. This is as a result of FERM and kinase domain interactions, causing FAK to remain in an auto-inhibited conformation[269], until clustering of integrin receptors at the cell membrane mediates binding of the cytoplasmic domains of integrins to the amino-terminal domain of FAK followed by FAK dimerization[270]. This leads to disassociation of the intra-molecular interactions mediating the auto-inhibitory configuration, and auto-phosphorylation of tyrosine residue 397 (Y397)[268]. Phosphorylated Y397 acts as a high affinity-binding site for Src, and on binding, results in a cascade of tyrosine residue phosphorylation (Y576, Y577, Y861 and Y925)[271]. Y397 and Y576/Y577 phosphorylation are essential for maximal catalytic activity, efficient disassembly of focal adhesions and therefore cell migration[272], whilst phosphorylation of the remaining tyrosine residues create high affinity binding sites for other SH2 domain containing proteins[273]. This results in the open conformation and fully activated FAK-Src signalling complex, capable of functioning as both a scaffold and as a kinase. Subsequent protein binding partners to this FAK-Src scaffold include PI3K/AKT[274], growth factor receptor bound protein 2 (Grb2), phospholipase C gamma (PLCγ)[275], the SH3 binding protein p130Cas[276], nuclear p53[277,278] and FAT binding proteins: talin[279] and paxillin[280] (**figure 1.3**) amongst others. FAK-FERM domain alterations can additionally be stimulated by changes in pH and increased cell–ECM tension, which can additionally increase the activity of FAK[281–283]. Force-mediated FAK activation promotes downstream signalling inducing mitotic spindle reorganisation[284], triggering mechano-sensitive cell proliferation[285], and can increase inflammatory cytokine production associated with fibrosis[286], as has been demonstrated by Jiang and colleagues in a murine models of pancreatic cancer where FAK inhibition resulted in a reduction in fibrosis[142]. The amino terminal FERM domain also mediates protein-protein interactions with the cytoplasmic tails of β1 integrins and complexes containing key signalling molecules including growth factor receptors such as PDGFR (platelet derived growth factor receptor) and EGFR (epidermal growth factor receptor)[287–289]. These interactions also release the FAK-FERM conformation mediating the auto-inhibitory conformation, resulting in activation of FAK.

A nuclear export signal (NES) is located within the kinase domain and another in the FERM domain, which also contains nuclear localization sequences (NLS) (**Figure**

1.3). These allow events at the cell periphery to be transmitted co-ordinately to the nucleus[267]. Under normal conditions FAK resides in the cytoplasm of cells with only a very small quantity present within the nucleus. When cells are placed under stress, such as oxidative stress, FAK can shuttle from a cytoplasmic position associated with FAs, accumulate in the nucleus, and can cause changes in gene expression of various proteins[290], as well as binding distinct nuclear protein complexes. This process is mediated by the NLS within the FERM domain[291]. Identification of FAK's ability to translocate to the nucleus is a relatively new finding, and whilst a growing number of nuclear binding partners are being identified[290,292,293], its full function within the nucleus or the method in which FAK translocates to the nucleus is not completely understood. TP53, a protein commonly deregulated in many cancer types including PDAC[31] is one such protein that nuclear FAK associates with. Nuclear FAK allows cell proliferation by binding to TP53 in the nucleus, leading to TP53 degradation and therefore cell survival[277]. Other FAK dependent changes at the nuclear level have also been described. For example FAK alters cytokine gene expression, one of which is the IL6 gene in endothelial cells[294]. More recently transcriptional regulation of chemokine/ cytokine expression by nuclear FAK in cancer has also been described[142,197].

A



B

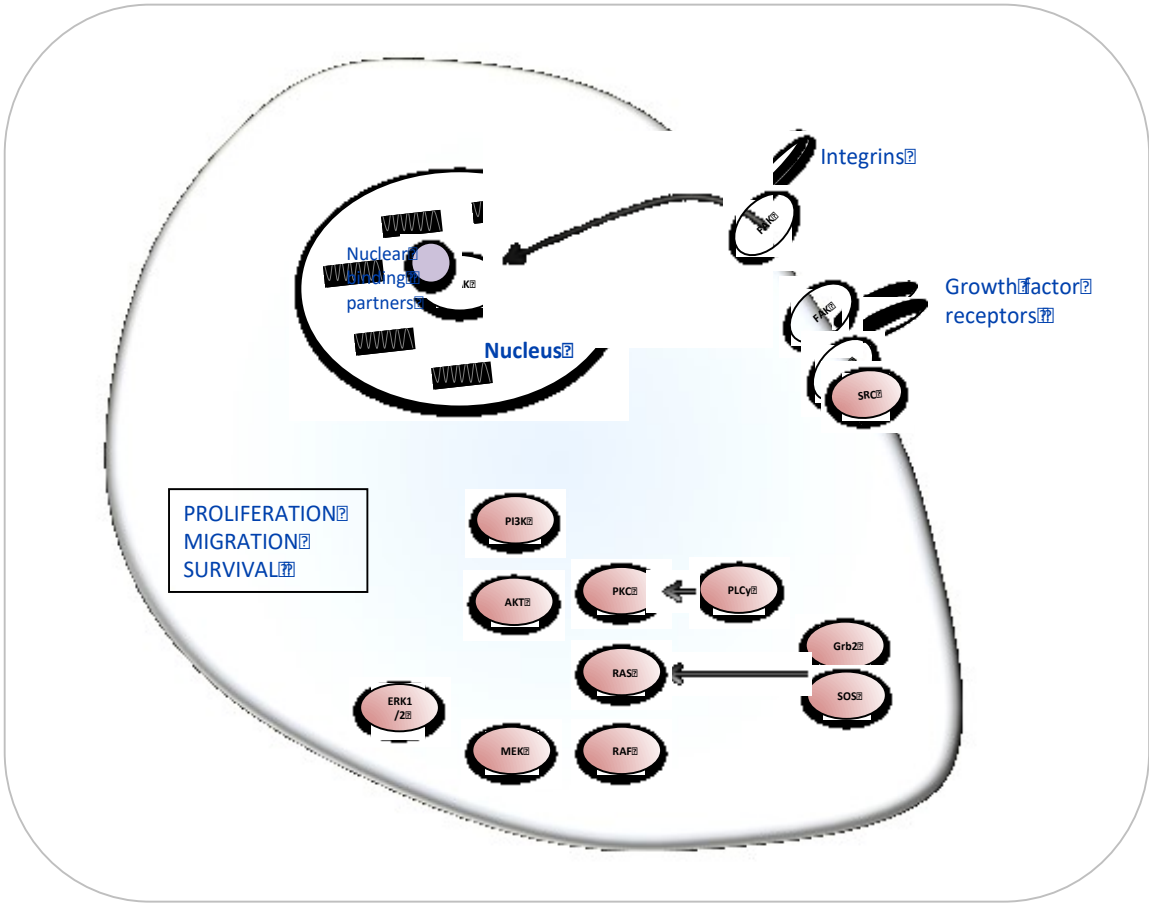


Figure 1.3 Basic structure and binding partners of FAK

A FAK is composed of a central kinase domain flanked by an N- terminal FERM domain and a C-terminal region containing a FAT domain. Proline-rich motifs (PR1, PR2 and PR3) link the central kinase domain with the terminal domains. The NLS is located in the F2 domain of FERM domain.
B FAK is a pivotal signal integrator that both controls and co-ordinates a number of cellular processes downstream of integrin and growth factor receptors. FAK interacts with and phosphorylates SRC. The

activated FAK/SRC complex directly or indirectly activates other kinases and shuttles to the nucleus where it interacts with nuclear binding partners to control cell proliferation, migration and survival.

1.3.2 FAK in cancer

FAK is central to multiple signalling pathways regulating various cellular processes and is over expressed in a number of cancer types including SCC of the skin, lung, head and neck, bladder carcinoma[263], breast[295–297], oral[298], prostate[299] and pancreatic[142,300] cancers amongst others. Therefore it is not surprising that FAK is implicated in cancer development and spread, and is considered a potential therapeutic target. FAK has been linked to the maintenance of cancer stem cells and has been implicated in promoting cancer cell survival, tumour growth, cell migration, invasion and angiogenesis[263]. Additionally, recent data has uncovered a novel role for nuclear FAK in controlling the composition of the immunosuppressive tumour microenvironment via FAK-dependent regulation of chemokines and cytokines. Using a syngeneic mouse model of SCC, FAK was found to translocate to the nucleus, where it interacts with a number of transcription factors and transcriptional regulators to enhance the expression of a variety of chemokines and cytokines[301]. CCL5 expression was up-regulated in FAK expressing SCC, resulting in elevated intra-tumoural Treg levels and suppression of the anti-tumour $CD8^+$ T-cell response. This lead to unregulated tumour growth. Inhibition of FAK by genetic depletion or use of a FAK inhibitor drug was found to reverse this effect. FAK has also been implicated in the regulation of other cytokines and chemokines in the context of cancer. Through TNF-alpha induced FAK activation of MAPK, IL6 transcription and protein translation was induced in breast, neuroblastoma, lung and prostate carcinoma[302].

1.3.3 FAK in PDAC

FAK is overexpressed in PDAC[142,300]. This increased expression positively correlates with increasing tumour size[300]. Two studies have indicated the potential for FAK inhibition in treatment of pancreatic cancer. Stokes et al demonstrated that the small molecule FAK/PYK2 inhibitor PF-562,271 inhibited *in-vitro* migration of human PDAC cells, murine CAFs and macrophages conditioned with tumour media. These findings were mirrored by their *in-vivo* findings whereby orthotopic implantation of PDAC cells into the murine pancreas resulted in reduced tumour proliferation,

invasion and metastasis when mice were treated with PF-562,271[303]. Jiang et al demonstrated administration of the small molecule FAK kinase inhibitor VS-4718 increased survival time in the KPC murine model of PDAC and found that FAK modulated a variety of chemokines and cytokines in the pancreatic TME. They specifically focused on CXCL12 and found this chemokine drives fibroblast proliferation and stromal expansion. FAK inhibition reduced the desmoplastic reaction within these tumours and altered the immune populations; they demonstrated reduced MDSCs, MMR^{+} macrophages, and Tregs levels[142]. Additionally they found that FAK inhibition synergised with immune checkpoint blockade to improve anti-tumour response and survival time. Tumour regression however was not observed in these studies. These encouraging pre-clinical results identify a potentially important role for FAK in regulating the composition of the immunosuppressive TME.

1.4 Hypothesis and Objective

Hypothesis

FAK is a key modulator of immune signalling in pancreatic cancer

Objective

To identify novel mechanisms of FAK-dependent immune regulation in pancreatic cancer

2 Materials and Methods

2.1 Material and methods (*in-vitro*)

2.1.1 Cell lines and cell culture

Panc47 cell lines were obtained from Jen Morton (The Beatson Institute for Cancer Research, Glasgow). This cell line was originally derived from the *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$; Pdx1 Cre (KPC)* mouse.

Panc47 and derivatives were cultured at 37ºC / 5% CO_2 in Dulbecco's Minimum Essential Medium - high glucose (DMEM - high glucose; *Sigma Life Technologies*) supplemented with 10% foetal bovine serum (FBS, *Sigma Life Technologies*). Cell lines were cultured in 25-, 75- and 150cm^2 plastic tissue culture flasks (*Cell Star, Greiner bio-one* Falcon vented cell culture flasks). When cells were near to 80% confluent, they were washed with phosphate-buffered-saline (*QMRI central services,* Sterile PBS), and treated with 1x trypsin (Gibco™, *Sigma Life Technologies*) diluted in sterile PE (*QMRI central services,* Sterile PE). Once cells had detached from the surface of the flasks the trypsin was quenched by the addition of fresh culture media containing FBS. Cells were then diluted to an appropriate ratio depending on the intended use. Stocks of all cell lines were frozen in 10% dimethyl sulfoxide (DMSO, *Sigma Life Technologies*) in DMEM supplemented with 20% FBS and stored at -196ºC in liquid nitrogen.

2.1.2 Generation of CRISPR model

Type II CRISPR/Cas9 genome editing technology was used to deplete FAK from Panc47 as described in the protocol published by Ran et al[304].

2.1.2.1 Cloning of gRNA oligonucleotides into the Cas9 vector plasmid

The online CRISPR design tool (http://crispr.mit.edu/) was used to generate two pairs of complementary 20bp length gRNAs: gFAK4 and gFAK6 (**figure 3.3**) with BbsI restriction enzyme overhangs to allow annealing into the expression plasmid.

Oligos were annealed by combining 1µl of forward and reverse sgRNA, 1µl T4 ligation buffer (*ThermoFisher Scientific*), 1µl T4 PNK (*New England Biolabs),* 6ul ddH_2O and incubated at 37ºC for 30 minutes; 95ºC for 5 minutes; 25ºC for 5 minutes. The annealed oligos were diluted 1:100 by adding 1µl oligo to 100µl room temperature ddH_2O. The guide expression plasmid pSPCas9(BB)-2A-GFP was chosen because it contains a GFP selection cassette to allow for easy identification of cell clones where retroviral transfection into target cells has been successful. The complementary guide oligonucleotides were annealed and cloned into a CRISPR guide expression plasmid pSPCas9(BB)-2A-GFP (PX458) by setting up ligation reactions combining 2µl diluted oligos, 100ng sPsCas9(BB)-2A-GFP, 2µl Tango buffer, 1µl 10mM DTT, 1µl 10mM ATP, 1µl FastDigest *Bbs*I, 0.5µl T7 ligase (*all ThermoFisher Scientific*) and ddH_2O up to 20µl. The ligation reaction was then incubated at 37ºC for 5 minutes and 21ºC for 5 minutes for 6 cycles. The expression plasmid was then transformed into chemically competent Top10 bacteria by adding 2µl of the resulting ligation mix to transform the expression plasmid into one shot® TOP10 chemically competent *E.coli* (*ThermoFisher Scientific*) containing ampicillin resistance. Plates were incubated overnight at 37ºC and 3 colonies from each plate were picked with a sterile pipette tip and incubated in 3ml LB (Lysogeny broth) with 100µg ml^{-1} ampicillin overnight at 37ºC in order to take advantage of selection using the ampicillin resistance gene within the plasmid. 1.5ml of culture medium was pelleted and DNA was extracted from bacterial colonies using QIAprep spin miniprep kit (*Qiagen*) as per manufacturer's instructions. DNA was sent for sequencing at the IGMM sequencing facility using the U6 forward primer. Sequences were aligned to determine if they contained the inserted oligos. Clones 1 of both gRNAs were positive **(figure 3.3)**.

2.1.2.2 Transfection into Panc47 cell lines

To generate FAK-depleted Panc47 cell clones, cells were transfected with the expression plasmids containing either the gFAK4 or gFAK6 guide sequences. First plasmid DNA was amplified and isolated using QIAGEN Plasmid Maxi Kit (*Qiagen*), as per manufacturer's instructions. In sterile, dark conditions, 2ml Opti-MEM (*ThermoFisher Scientific*) was combined with 20µl lipofectamine® 2000 (*ThermoFisher Scientific*). 2ml Opti-MEM was combined with 5µg gFAK DNA in a

separate sterile container. After 20 minutes of room temperature incubation, these were combined and left to incubate for a further 10 minutes. Cell culture media was removed from pancreatic cell lines to be transfected and replaced with transfection media for 5 hours. After 5 hours, this was then replaced with normal pancreatic culture media. After 24 hours, these cell lines were visually checked for GFP expression using fluorescent microscopy and split as normal (1 in 10). 7 days later, isolation of positively transfected cells via GFP expression was performed with FACS (Fluorescence-activated cell sorting) sorter FACSAria II (*BD Biosciences).* Cells were dissociated with 1ml 2.5% trypsin solution (*Sigma Life Technologies*), passed through a 70µm cell strainer (*Becton Dickinson*) to create a single cell suspension, pelleted at 1000rpm for 5 minutes and re-suspended in 500µl FACS buffer. Cells were sorted into a 96-well plate containing normal pancreatic culture media supplemented with Penicillin-Streptomycin (*Gibco Life Technologies* 10,000U/mL, diluted 1:100).

Once confluent, GFP positive cell lines were transferred into flasks. Resulting cell colonies were tested for successful depletion of FAK expression using anti-FAK western blotting. From a total of 16 colonies tested, 12 were identified to be FAK deficient (**figure 3.4**).

2.1.2.3 Identification of the genetic mutation arising from CRISPR genome editing

One FAK depleted clone (47-g4-clone2 hereafter referred to as FAK-/-) was selected, genomic cDNA was extracted and PCR amplified using FAK specific primers. Genomic cDNA from the successfully created FAK-depleted line FAK-/- (identified via western blot) was isolated using Qiagen DNeasy Blood & Tissue Kit (*Qiagen*) as per manufactures instructions. Primer sequences flanking the genomic region targeted by the guide sequences were designed and used to PCR amplify a genomic DNA fragment.

Primers:
>FAK 740F
5'-TGCCTTTCCTAGTTGTCTCTCC-3'

>FAK 1053R
5'-TGTTTGTGAATAAGATTAAGGCCATC-3'

200ng of genomic cDNA was combined with 1.25µl of 10µM FAK 740 Forward primer, 1.25µl of 10µM FAK 1053 Reverse primer, 25µl NEB Phusion master mix (2x stock solution) and made up to 50µl sterile water in a 0.2µl PCR tube. PCR conditions as detailed below

	Temperature (°C)	Time (minutes)
Hot start	95	10
Number of cycles	40	
Denature	95	0.5
Anneal	62	0.5
Elongation	72	1
End cycle	_	_
Finish	72	7
Storage	4	

Using the FAK forward and reverse primers again, direct sequencing of this PCR product was performed at the IGMM sequencing facility to confirm depletion of FAK and identify the mutation that had occurred (**figure 3.5**). Sequences were aligned using **ClutsalW (European Bioinformatics Institute)** software, identifying a 4 base pair deletion at position 579 of the cds (complete coding sequence) (**figure 3.5A**). Comparison of the predicted protein sequence with that of wild-type FAK identified a premature STOP codon at AA position 193 (**figure 3.5B**) confirming that if a protein were to be produced, it would result in a severely truncated protein produced with a predicted molecular weight of approximately 22.4KDa. This protein

is unlikely to be functional or stable and a protein of this size was not detected on WB using an N terminal FAK specific antibody (**figure 3.6A**).

2.1.3 FAK re-expression

To generate a cell model with which to further study FAK function, I re-expressed FAK into Panc47 FAK-/- cells using retroviral transduction, thus creating identical FAK positive and negative clones.

Phoenix Ecotropic cells were transfected with a pWZL FAK-wt construct, previously used in the lab, using lipofectamine® 2000 (*ThermoFisher Scientific*) and Opti-MEM (*ThermoFisher Scientific*) as described in **2.2.2.2**. The FAK protein within the pWZL FAK-wt construct was originally derived form chicken. Mouse and chicken FAK polypeptides have been shown to be structurally similar throughout their lengths[305] as detailed below in appendix 2.

After 24 hours, virus-containing media was collected from phoenix ecotropic cells. This media was filtered through a 0.45μM Millex-HA filter (*Merck Millipore*), supplemented with 5μg/ml polybrene and applied to FAK-/- cells and cultured at 37ºC/ 5% CO_2 in Dulbecco's Minimum Essential Medium - high glucose (DMEM - high glucose; *Life Technologies*) supplemented with 20% foetal bovine serum (FBS, *Life Technologies*). After 24 hours a second round of infection was performed. Following the second round of infection, cells were cultured at 37ºC/ 5% CO_2 in DMEM supplemented with 10% FBS and maintained under selection using 0.25mg/ml hygromycin (*Merck Millipore*). Successful re-expression of FAK-wt was confirmed using western blotting (**figure 3.6A**). Biochemical fractionation to separate nuclear and cytoplasmic extracts followed by western blotting of protein extracts was carried out using anti-FAK antibodies **(figure 3.6B)** and FAK was found to be readily detectable in the nucleus of Panc47 FAK-wt cells.

2.1.4 shRNA (short hairpin) mediated IL6 knockdown

Lentiviral particles for infection of FAK-wt cells were generated by transfecting $2x10^6$ HEK23FT cells with a mixture of 2.5μg of each pLKO IL6 shRNA; TRCN0000067548, TRCN0000067549, TRCN0000067550, TRCN0000067551, TRCN0000067552 (RMM4534-EG16193, *Dharmacon*), or non targeting control

pLKO-NTCO (kind gift from Sonja Vermeren), 2.5μg of the viral packaging vector (psPAX2) and 2.5μg of the viral envelop (pMD2.G) mammalian expression packaging vectors (*Addgene*), 5ml Opti-MEM^{TM} media (*Thermofisher*) with Lipofectamine 2000 (*Thermofisher*) as per manufacture's protocol. Transfected HEK23FT cells were incubated for 8 hours at 37^{o}C, media was removed and replaced with media optimal for the target cell (FAK-wt): DMEM- high glucose supplemented with 20% FBS. After a further 24 hours of culture, media was removed and filtered through a 0.45μM Millex-AC filter (*Millipore*), supplemented with polybrene to a final concentration of 5μg/ml and added to the FAK-wt cells for 24 hours. Cells were subject to two rounds of lentiviral infection prior to selection with puromycin at a final concentration of 2μg/ml.

2.1.5 Re-expression of NLS mutant and KD mutant

Retroviral particles for infection of the FAK-/- cell line were generated by transfecting 2x10^{6} Phoenix ecotropic (Phoenix-Eco, *Thermofisher)* cells with a mixture of 3μg vector (NLS mutant, KD mutant or PWZL empty vector control), 5ml Opti-MEM^{TM} media (*Thermofisher*) with Lipofectamine 2000 (*Thermofisher*) as per manufacture's protocol. Transfected Phoenix-Eco cells were incubated for 24 hours at 37^{o}C, media was removed, replaced with media optimal for the target cell (FAK-/-): DMEM-high glucose supplemented with 20% FBS. After a further 24 hours of culture media was removed and filtered through a 0.45μM Millex-AC filter (*Millipore*), supplemented with polybrene to a final concentration of 5μg/ml and added to the FAK-/- cells for 24 hours. Cells were subject to two rounds of infection prior to selection with hygromycin at a final concentration of 10μg/ml.

2.1.6 IL6 re-expression into FAK-/-

Lentiviral particles for infection of the FAK-/- cell line were generated by transfecting 2x10^{6} HEK23FT cells with a mixture of 5μg IL6 vector (IL6 supplied in pLenti-C-Myc-DDKIRES-GFP vector, MR227281L2, *OriGene Technologies*), 2.5μg of each of the viral packaging (psPAX2) and viral envelop (pMD2.G) mammalian expression packaging vectors (*Addgene*), 5ml Opti-MEM^{TM} media (*Thermofisher*) with Lipofectamine 2000 (*Thermofisher*) as per manufacture's protocol. Transfected HEK23FT cells were incubated for 8 hours at 37^{o}C, media was

removed, replaced with media optimal for the target cell (FAK-wt): DMEM- high glucose supplemented with 20% FBS. After a further 24 hours of culture, media was removed and filtered through a 0.45μM Millex-AC filter (Millipore), supplemented with polybrene to a final concentration of 5μg/ml and added to the FAK-wt cells for 24 hours. Cells were subject to two rounds of lentiviral infection prior to selection with chloramphemicol at a final concentration of 34μg/ml.

2.1.7 Western blot

Plated cells were washed twice with chilled PBS and lysed in radioimmuno-precipitation assay (RIPA) buffer (50mmol/L Tris (pH 7.6), 150mmol/L sodium chloride, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS) supplemented with phosphatase inhibitor cocktail (*Roche*) and protease inhibitor cocktail (*Roche*). Lysates were cleared by centrifugation at 13000 rpm for 15 minutes at 4ºC. Protein concentration was determined using Pierce™ BCA protein Assay kit (*Thermo Fisher*). Absorbance was measured with a Beckman DU 650 spectrophotometer (*Beckman Coulter, Luton, UK*) and read at 562nm wavelength. 20μg of each sample was added to 10μl 2x sample buffer and made up to a total of 20μl with RIPA buffer. Lysate mixtures were then incubated at 95ºC for 10 minutes. 2μl of pre-stained protein ladder Precision plus protein™ dual color standard (*biorad cat 161-0374*) was used as MW marker. Lysates were resolved by 4-15% Bis-Tris gel electrophoresis (*Biorad*) and proteins transferred to nitrocellulose membranes using the Trans-Blot® Turbo™ Transfer System (*Biorad*). Membranes were then blocked (5% BSA/TBST) for 1 hour at room temperature and probed with relevant primary antibody: anti-FAK antibody (1:1000, clone 4.47 in 5% BSA/TBST; *Millipore*) or FAK pY397 antibody (1:1000, 3283; *Cell Signalling Technology*, in 5% BSA/TBST) overnight at 4ºC. Membranes were then washed three times with TBST buffer (Tris Buffered Saline with Tween) and bound antibody was detected by 1-hour room temperature incubation with anti-mouse or anti-rabbit HRP-conjugated secondary antibody (1:5000 in 5% BSA/TBST; *Cell Signaling Technology*). Membranes were washed three times with TBST and prepared for chemiluminescent visualisation by incubation in Clarity™ Western ECL Blotting Substrate (*Biorad*). Membranes were visualised using a ChemiDoc MP Imaging System (*Biorad*). Membranes were then washed with distilled water at room temperature for 20 minutes and stripped with

Re-Blot Plus Strong Antibody Stripping solution (*Merek Millipore*). Membranes were washed twice with distilled water and blocked (5% BSA/TBST) for 1 hour at room temperature. Stripped membranes were then re-probed with anti-GAPDH (1:10000 in 5% BSA/TBST, *Cell Signaling Technology*) or anti-tubulin (α-tubulin, clone DN1A, *Cell Signalling Technology*) overnight at 4°C to assess equal protein loading or to assess efficient fractionation for western blots following nuclear fractionation. Membranes were then washed three times with TBST and bound antibody was detected by 1-hour room temperature incubation with anti-mouse or anti-rabbit HRP-conjugated secondary antibody (1:5000 in 5% BSA/TBST; *Cell Signaling Technology*). Membranes were washed three times with TBST and prepared for chemiluminescent visualisation as before. For western blots following nuclear fractionation, membranes were again stripped as before and washed twice with distilled water. Again membranes were blocked (5% BSA/TBST) for 1 hour at room temperature. Stripped membranes were then re-probed with anti-PARP (1:10,000 in 5% BSA/TBST, *Cell Signaling Technology*) primary antibody overnight to check for efficient fractionation. Membranes were washed three times with TBST and prepared for chemiluminescent visualisation as described above.

2.1.8 Nuclear fractionation

Plated Panc47, FAK-wt, FAK-/- or FAK-NLS cells were washed twice with chilled PBS and lysed in CYTO buffer (10 mM Tris-HCl pH 7.5, 0.05mM NP-40**,** 3mM $MgCl_2$, 100mM Sodium Chloride (NaCl), 1mM EGTA (*Sigma Chemical Co*), phosphatase inhibitor cocktail (*Roche*) and protease inhibitor cocktail (*Roche*). Lysates were centrifuged at 13000 rpm for 15 minutes at 4°C to separate the nuclear pellet from the cytoplasmic fraction. The cytoplasmic fraction was then transferred into a new tube and an equal volume of 2x RIPA buffer added. The nuclear pellet was washed twice in CYTO buffer and centrifuged at 1000rcf. Remaining CYTO buffer was aspirated and purified nuclear pellets were lysed in 150µl RIPA buffer for 15 minutes at 4°C and centrifuged at 16000 rpm for 15 minutes to separate the non-soluble portion of the nuclear fraction. The supernatant was transferred into a new tube. Protein concentration was then determined using Pierce™ BCA protein Assay kit (*Thermo Fisher*). Absorbance was measured with Synergy 2 Multi-Mode Reader

(*BioTek®, Swindon, UK*) and read at 562nm wavelength. Western blot performed as described above.

2.1.9 Enzyme-linked immunosorbent assay (ELISA)

Cell lines were counted and plated equally into 6 well plates (*costar®*). After 24 hours, media was changed and appropriate amount of mouse IL17 recombinant protein (*eBioscience*) containing media was applied to plates. Plates were then incubated at 37ºC / 5% CO_2 for 48 hours. Media was collected and ELISA assay carried out using mouse IL6 DuoSet ELISA kit (*R&D systems*) according to the manufacturer's instructions. The samples were analysed with a plate reader by optical density (OD) at a wavelength of 450nm (Synergy 2 Multi- Mode Reader, *BioTek®*). Readings were conducted in duplicate. Plates were washed twice with chilled PBS and protein concentrations were determined in order to normalise ELISA results using Pierce™ BCA protein Assay kit (*Thermo Fisher*).

2.1.10 Flow cytometry of cultured cells

Cells were cultured in 75cm^2 Cell Star® cell culture flasks (*Greiner bio-one*). Cells were removed using 1x non-enzymatic cell dissociation solution (*Sigma Life Technologies*) and pelleted by centrifugation at 1600rpm for 5 minutes at 4ºC. Cell pellets were re-suspended in PBS and passed through a 70µm cell strainer (*Becton Dickinson*) to create a single cell suspension. This was then pelleted by centrifugation at 1600rpm for 5 minutes at 4ºC and this PBS wash was repeated before transferring 200µl of appropriate sample into each well of a 96-well round-bottomed plate. The plate was centrifuged at 1600rpm for 5 minutes at 4ºC. Cell pellets were re-suspended in 200µl PBS (unstained control), and the rest in 200µl of Zombie NIR™ (fixable viability dye, *Biolegend*), diluted 1:1000 in PBS. Plates were sealed and incubated at 4ºC for 30 minutes in the dark. Plates were then centrifuged at 1600rpm for 5 minutes at 4ºC and re-suspended in FACS buffer (Phosphate-buffered saline solution (PBS) supplemented with 2% heat-inactivated FCS (*Labtech*) and 0.1% sodium azide (*Sigma*). This step was repeated for a total of 3 times. Cell pellets were then re-suspended in 100µl of Fc block (1:200 dilution of Fc antibody (*eBioscience*) in FACS buffer) and incubated for 15 minutes at room temperature in the dark. Into the appropriate samples, 100µl of antibody was added: either IL-17A receptor antibody (anti-mouse CD217 (IL-17 Receptor A) PE,

eBioscience), or isotype control (anti-mouse IgG conjugated to PE, *eBioscience*), or antibodies as described in STAIN 1, 2, and 4 (**tables 4.1, 6.1 and 6.2**). Appropriate FMOs (fluorescence minus one) controls for gating were also created at this time. All antibodies were made up at a concentration of 1 in 200 FACS buffer giving a final concentration of 1:400. Plates were incubated for 30 minutes in the dark at 4ºC. Plates were then centrifuged at 1600rpm for 5 minutes at 4ºC and re-suspended in FACS buffer. This step was repeated for a total of 3 times. Cell pellets were then re-suspended in FACS buffer and analysed using a BD FACS Aria II (*Becton Dickinson*). Data analysis was performed using FlowJo software. Statistics and graphs were calculated using Prism (*Graphpad*).

2.1.11 Forward-Phase Protein Arrays (FPPA)

FAK-wt and FAK-/- cell lines were trypsinised, counted and 0.25 x 10^6 cells were added to each well of a 6 well plate. After 24 hours, media was changed and these cells were cultured at 37ºC / 5% CO_2 in Dulbecco's Minimum Essential Medium - high glucose (DMEM - high glucose; *Life Technologies*) supplemented with 1% foetal bovine serum (FBS; *Life Technologies*). Media was collected for FPPA analysis after 48 hours of incubation. Microarrays were generated using the in-house Aushon BioSystems' 2470 array printing platform. In order to normalise array results, plates were washed twice with cold PBS, protein lysates made and protein concentration was determined using Pierce™ BCA protein Assay kit as per manufacturers instructions (*Thermo Fisher*). Microarrays were blocked for 1 hour with SuperG™ Blocking Buffer (*Grace Bio Labs*) at room temperature on a rocker. Media from samples were centrifuged at 1000rcf for 5 minutes at 4ºC. Supernatants were added to microarrays for 12 hours at 4ºC. Microarrays were washed three times for 5 minutes in TBST, and blocked for 10 minutes with SuperG™ Blocking Buffer at room temperature on an orbital shaker, then washed again washed three times for 5 minutes in TBST. Detection antibodies (1:500 antibody diluted in 5% BSA/PBST, 1% SuperG™ Blocking Buffer) mixtures were made in plates and 2µl of each antibody was applied to each well of the microarrays. Microarrays were clamped and 50µl of each antibody was added to corresponding microarray wells. Microarrays were incubated for 1 hour on a flat surface. Clamps were removed and microarrays were washed three times for 5 minutes in TBST. Microarrays were then blocked for

10 minutes with SuperG™ Blocking Buffer at room temperature on a rocker and again washed three times for 5 minutes in PBST. 3ml of IRDye® 800CW Streptavidin (*LI-COR Biosciences*) diluted 1 in 5000 in PBST supplemented with 5% BSA, 1% SuperG™ Blocking Buffer. Microarrays were covered and incubated on a rocker at room temperature for 45 minutes then washed for 5 minutes, three times in PBST followed by three 5 minute PBS washes and then washed with distilled water. Microarrays were dried then scanned on the InnoScan 710 high resolution Microarray scanner (*Innopsys Life Sciences*). Data was normalised for protein concentration and background fluorescence in Microsoft Excel. Graphs were calculated using Prism (*Graphpad*).

2.1.12 Optimisation of *BI853520* dose

I undertook a series of experiments utilising the FAK kinase inhibitor *BI853520*, a drug currently in clinical development[306,307]. It was necessary to first optimise the dose of *BI853520* required to selectively inhibit FAK kinase in my pancreatic tumour cell line *in-vitro*.

A dose response experiment was carried out to establish the dose required to inhibit FAK phosphorylation, indicating successful FAK kinase inhibition *in-vitro*. FAK-wt cells were treated with escalating doses of *BI853520* ranging from 0.25nM to 250nM diluted in DMEM- high glucose (*Sigma Life Technologies*). Western blotting was performed as described in 2.2.7 however fluorescent secondary antibodies: DyLight™ 800 or DyLight™ 800 Conjugate (*Cell Signalling Technology; #5366*) were used in place of HRP-conjugated secondary antibody and fluorescent intensity was determined using the Licor fluorescent imager.

2.1.13 *In-vitro* phenotypical characterisation of BMDMs cultured in FAK-wt conditioned media

Bilateral tibias and femurs dissected from C57BL/6 mice were flushed with 5ml of DMEM medium supplemented with 10% FBS and 1% Penicillin/Streptomycin into a 50 ml tube, washed in medium once and filtered through a 70µm cell strainer (*Becton Dickinson*). Cells were seeded at 1 x 10^6 per well in a 6 well plate and cultured in 2ml of DMEM with 10% FCS and 25ng/ml recombinant mouse M-CSF (*Thermofisher*). Cells were incubated for 7 days then washed with PBS followed by

replacement with fresh media (DMEM medium supplemented with 10% FBS) or conditioned media from FAK-wt cells cultured for 48 hours with/without the addition of 20ng/ml recombinant mouse IL4 (*Thermofisher*), or fresh media with recombinant mouse G-CSF, GM-CSF or IL6 (*Thermofisher*). All additional cytokines were added at 20ng/ml. Cells were cultured for a further 24 hours, washed with PBS and harvested using non-enzymatic dissociation buffer, incubated with an Fc block, and stained for surface receptors using flow cytometry (**2.2.10**).

2.1.14 CD4$^+$ T-cell polarisation

Splenocytes were obtained by mechanical disaggregation of the tissue (between layers of sterile gauze) and re-suspended as a single-cell suspension in wash buffer (HBSS; *Sigma*). For all wash steps, cells were centrifuged at 1600rpm for 5 minutes. Red blood cells (RBCs) were lysed at room temperature for two minutes using RBC lysis buffer (*Sigma*).

Cells were then washed in MACS buffer (HANKS balanced salt solution supplemented with 2% heat-inactivated FCS, *Sigma*) and CD4$^+$ T-cells isolated from splenocytes with CD4 microbeads (*Miltenyi*) using the autoMACS®Pro Separator as per manufacturers instructions. Cells were re-suspended in RMPI and seeded at 2 x 10^5 cells in a 48 well plate and cultured in 1ml of FAK-wt or FAK-/- RMPI conditioned media (RMPI with 20% FCS, 1x L-Glutmaine, 1x Pen/Step, 80 U/ml IL2 and β-50 μM mercaptothanol). Cells were harvested after 24, 48 72 and 96 hours.

2.2 Material and methods (*in-vivo*)

2.2.1 Mice

Female C57BL/6 mice (*Charles River; Kent, UK*) were used for all *in-vivo* experiments.

All mice were supplied as age matched, 5-week old females and isolated for one week after delivery. All experiments had University of Edinburgh ethical approval and were carried out in accordance with the United Kingdom Animal Scientific Procedures Act (1986).

2.2.2 Orthotopic implantation of cancer cells into the pancreas

Mice were anaethestised with inhalational isoflurane anaesthetic in oxygen, and received perioperative analgesia: buprenorphine (*Vetergesic, 0.1mg/kg s.c*) and carprofen (*Rimadyl, 10mg/kg s.c*) and also post surgery, once daily for 48 hours. Cell lines were propagated to sub-confluency to ensure they were in their exponential growth phase. Once detached from the flask and washed with PBS, $0.5x10^6$ cells of the appropriate cell line was suspended in growth factor reduced matrigel basement membrane matrix (*Scientific Laboratory Supplies Ltd*.), at a concentration of $0.5x10^6$ cells in 25µl. Using aseptic technique, a 3mm skin inscision was made in the left lateral flank and lateral abdominal muscles in order to visualise the pancreas. $0.5x10^6$ cells in 25µl matrigel were injected into the pancreas in a sterile manner. The abdominal wall was closed with Polyglactin 910 (*Vicryl, 2M; Henry Schein*), with a single cruciate suture. Skin was closure was optimised. Skin clips and *Vicryl* sutures were compared and skin clips were deemed superior with regards to closure time and post-operative mouse interference. Mice were monitored in a heat box set to 37ºC post surgery for 1 hour. Mice were closely monitored daily with twice weekly weight checks following implantation. If any one terminal symptom caused by pancreatic tumour growth including weight loss equal or exceeding 10% of the starting weight, signs of abdominal pain or abdominal distension became apparent, the animal was humanely euthanized. After two weeks, the animals were culled (cervical dislocation) and the pancreatic tumours were harvested for analysis.

2.2.3 Flow cytometry analysis of tissue

Tumour tissue was mashed into a pulp using a scalpel and re-suspended in DMEM (*Life Technologies*) supplemented with 2mg/ml collagenase D (*Roche*) and 10µl/ml DNase 1 (*Roche*). Samples were incubated for one hour at 37ºC, shaking at 150rpm. Samples were then pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in 5ml of 1x red blood cell lysis buffer (*Pharm Lysis Buffer; Becton Dickinson*) followed by incubation at 37ºC for 10 minutes. Samples were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in PBS then passed through a 70µm cell strainer (*Becton Dickinson*) to create a single cell suspension. Cells were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC,

and re- suspended in PBS before pipetting 100µl of each sample into well of a 96-well plate. The plate was then centrifuged at 1600 rpm for 5 minutes at 4ºC. Cell pellets were re-suspended in 200µl PBS (unstained control), and the rest in 200µl of Zombie NIR™ (fixable viability dye; *Biolegend*), diluted 1:1000 in PBS. Plates were sealed and incubated at 4ºC for 30 minutes in the dark. Samples were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in FACS buffer (PBS + 1% FBS + 0.1% sodium azide; *Sigma*). This wash step was repeated. Samples were re- suspended in 100µl Fc block (CD16/32; *eBioscience*) diluted 1 in 200 in FACS buffer. Samples were incubated for 15 minutes at 4ºC. 100µl of surface staining antibody mixture was then added to each well, apart from FMOs and unstained control, and the samples incubated for 30 minutes in the dark at 4ºC. All antibodies were used at a concentration of 1 in 200 diluted in a 50:50 mix of Fc block and BD Horizon™ Brilliant Stain Buffer, giving a final concentration in the well of 1 in 400. The samples were incubated for 30 minutes in the dark at 4ºC. Samples were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in FACS buffer. This wash step was repeated. Intracellular transcription factor FoxP3 staining was performed using a Treg staining kit (*Biolegend*). Samples were re-suspended in 200µl 1x FoxP3 Fix/Perm Buffer made up as per manufacturers instructions. Samples were incubated for 12 hours at 4ºC in the dark. Samples were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in 1x FoxP3 Perm buffer. This wash step was repeated. Samples were re-suspended in 100µl Fc block (CD16/32; *eBioscience*) diluted 1 in 200 in FACS buffer. Samples were incubated for 15 minutes at 4ºC. 100µl of FoxP3 antibody mixture was then added to each well, apart from FoxP3 FMO and the unstained control. The samples were incubated for 30 minutes in the dark at 4ºC. All antibodies were made up at a concentration of 1 in 100, giving a final concentration 1 in 200 in the well. Samples were pelleted by centrifugation at 1600 rpm for 5 minutes at 4ºC, and re-suspended in FACS buffer and transferred into sterile 1.2ml Microtubes (*StarLabs*), pre-filled with a further 200µl FACS buffer. Samples were analysed using a BD FACS Aria II (*Becton Dickinson*). Data analysis was performed using FlowJo Software. Graphs were calculated using Prism (*Graphpad*).

2.2.4 $CD4^+/CD8^+$ antibody depletion

T-cell depletion was achieved following intra-peritoneal (IP) injection of 150μg of anti-mouse CD4 (GK1.5, ATCC TIB-207), anti-mouse CD8 (2.43, ATCC TIB-210) depleting antibodies or Rat IgG isotype control (all *eBioscience*) into female, age matched C57BL/6 mice for two consecutive days. Following two days of no treatment, mice were surgically implanted with $0.5x10^6$ cells of the appropriate cell line. IP antibody administration was repeated again on the day of surgery, days 8, 12 and 15 (**figure 4.2A**). Mice were culled two weeks after surgery and pancreatic tumours harvested for analysis.

2.3 Statistics

Statistical analysis was carried out using Graph Pad Prism7 for Mac (*Graph Pad Software*). All error bars on graphs represent standard error of the mean (SEM). Statistical tests used were analysis of variance (ANOVA) with Tukey's multiple comparisons test and paired T-test. P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

2.4 List of antibodies

MARKER	FLUOROPHORE	SUPPLIER	CLONE
Ly6G	BUV395	BD Horizon	1A8
CD140a	BV650	BD bioscience	APA5
F480	BV421	Biolegend	BM8
CD11b	BV510	Biolegend	M1/70
CD31	BV605	Biolegend	390
PD-L1/CD274	BV711	Biolegend	10F.7G2
Ly6C	PerCP/Cy5.5	Biolegend	HK1.4
MMR	PE	Biolegend	C068C2
CD11c	PE/DAZZLE	Biolegend	N418
CD86	PE/Cy7	Biolegend	GL-1
PD-L2/CD273	APC	Biolegend	TY25
CD45	AF700	Biolegend	30-F11
CD4	BV510	Biolegend	GK1.5
CD4	BV650	Biolegend	RM4-5
CD25	BV650	Biolegend	PC61
FoxP3	AF647	Biolegend	MF-14
GATA3	PE	E-bioscience	TWAJ
Viability	Zombie NIR	Biolegend	Cat 423105

Table 2.1 List of antibodies, clones and suppliers for stains 1, 2 and 3

3 Generation of a FAK-/- and FAK-wt PDAC cell line

3.1 Introduction

Recent studies using murine models of pancreatic cancer have identified a potentially important role for FAK in regulating the composition of the immuno-suppressive tumour microenvironment (TME)[142,303]. Mechanisms underpinning this regulation however are poorly understood. I therefore set out to use genetic manipulation of pancreatic cancer cells in order to investigate the role of cancer-cell intrinsic FAK signalling in modulating pancreatic tumour growth and the immuno-suppressive TME.

Genome sequencing has identified that the most commonly mutated genes in human PDAC are KRAS, TP53, CDKN2A, SMAD4, and transforming growth factor-β (TGF-β) pathway alterations[35,308]. Although activation of oncogenic KRAS alone in pancreatic epithelial cells is sufficient to initiate PDAC in murine models, additional mutations in TP53, CDKN2A or SMAD4 can accelerate PDAC progression[309]. Thus there are now a number of transplantable and genetically engineered mouse (GEM) models routinely used in pre-clinical pancreatic cancer research, developed to incorporate one or more of these mutations whilst reliably emulating many features of this disease in humans. Patient derived cell lines and organoid-based xenograft models[309] are also available but these require implantation into immune compromised hosts and are therefore not suitable for the purpose of my study. GEM models currently available are listed in **table 3.1**[310,311,320,321,312–319]

TABLE 3.1 GEM MODELS OF PDAC	
GEM MODEL	**REFERENCE**
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; p53$^{R172H/+}$(KPC)	Hingorani et al., 2005
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; p53$^{lox/lox}$	Bardeesy et al., 2006
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; p53$^{lox/lox}$Ink4a/Arf$^{-/-}$	Bardeesy et al., 2006
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Smad4$^{lox/lox}$	Bardeesy et al., 2006
Ptf1a-Cre; K-Ras$^{+/LSLG12D}$; TGFβIIR$^{lox/lox}$	Ijichi et al., 2006
Elastase-tTA; Tet-O-Cre; K-Ras$^{+/LSLG12Vgeo}$; p53$^{-/-}$	Guerra et al., 2007
Ptf1a-Cre; K-Ras$^{+/LSLG12D}$; Elastase-TGF-a	Siveke et al., 2007
Ptf1a-Cre; K-Ras$^{+/LSLG12D}$; Smad4$^{lox/lox}$	Izeradjene et al., 2007
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Brca2$^{Tr/\Delta 11}$	Skoulidis et al., 2010
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; p53$^{R270H/+}$; Brca2$^{Tr/+}$	Skoulidis et al., 2010
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; p53$^{R270H/+}$; Brca2$^{Tr/\Delta 11}$	Skoulidis et al., 2010
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Lkb1$^{lox/lox}$	Morton et al., 2010
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Notch1$^{lox/lox}$	Hanlon et al., 2010
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Notch2$^{lox/lox}$	Mazur et al., 2012
Elastase-tTA; Tet-O-Cre; K-Ras$^{+/LSLG12Vgeo}$; p53$^{lox/lox}$	Guerra et al., 2011
Elastase-tTA; Tet-O-Cre; K-Ras$^{+/LSLG12Vgeo}$; Ink4a/Arf$^{lox/lox}$	Guerra et al., 2011
Pdx1-Cre; K-Ras$^{+/LSLG12D}$; Usp9x$^{+/lox}$	Perez-Mancera et al., 2012
Mist1^{Cre}ERT2/+; LSL- *Kras*^{G12D}	Habbe et al., 2008
Trp53-null CPB2$^{wt/11}$	Rowley et al., 2011

Table 3.1 List of current GEM models of PDAC[322,323]

The *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$; Pdx1 Cre (KPC)*[310] GEM model of pancreatic cancer is regarded as one of the best autochthonous murine models of pancreatic cancer currently available. This is for a number of reasons, firstly, it harbours an activating mutation in KRAS and an inactivating or gain-of-function mutation in the tumour suppressor p53[18,35]. These reflect two of the most frequent mutations identified in human pancreatic cancers; point mutations in the KRAS2 proto-oncogene are found in 80-90% of PDACs, and point mutations in TP53 in 50-75% [310]. Secondly, the model accurately reproduces stages of disease progression in human PDAC, progressing from pancreata devoid of neoplastic cells in new born

mice to early pre-invasive lesions (PanIN 1,2 and 3) by 8-10 weeks, and to full PDAC by 4 – 5 months of age[324]. KRAS mutation rate increases in frequency as disease stage advances with TP53 mutations generally found in later stages of disease progression[26] (**figure 1.1**). Thirdly, the KPC model reproduces many of the major characteristics observed in human PDAC including significant infiltration of $F480^{+}$ macrophages, an intense desmoplastic reaction and exclusion of cytotoxic T-cells[324]. Lastly, it the most comprehensively evaluated genetic mouse model of PDAC for studying immunotherapies, exhibiting similar responses to that reported in man[325–328]. I therefore opted to use a syngeneic cell line isolated from PDAC arising on the KPC model as the starting point for my studies.

The goal of this work was to interrogate the role of cancer cell intrinsic FAK signalling in regulating pancreatic tumour growth and the immuno-suppressive TME. Therefore, it was necessary to use targeted deletion of FAK expression specifically in pancreatic cancer cells. A number of technologies are available for the genetic manipulation of cells, including CRISPR/Cas9 genome editing technology, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). CRISPR/Cas9 genome editing is the most recently developed method. Due to it's reliance on simple base-pairing between the PAM preceded target DNA with the sgRNA, CRISPR/Cas9 based gene editing is now widely used as an efficient and reliable tool for knocking out a broad spectrum of genes from various cell lines. The design and engineering of sequence-specific DNA binding proteins for ZFNs and TALENS remains costly, time-consuming and experimentally complex and therefore CRISPR/Cas9 gene editing has largely superseded these other nuclease gene editing tools[329]. The speed, efficiency, low cost and ease of CRISPR/Cas9 gene editing technology[329] represented an attractive option for rapidly creating a FAK depleted PDAC cell line.

The type II clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided Cas9 nuclease system is a programmable nuclease based genome editing technology. The technology was developed from naturally occurring viral host defence mechanisms within various bacterial species that identify and degrade invading foreign nucleic acids[330,331]. The system works by incorporating invading viral DNA into the bacterial host genome within its CRISPR arrays. This enables

host bacteria to recognise and destroy the invading pathogenic DNA upon reinfection. This bacterial defence mechanism is harnessed in CRISPR/Cas genome editing and CRISPR constructs can be designed to recognise and nuclease cleave targeted areas of DNA within the target mammalian cell genome. After cleaving specific sites, non-homologous end joining (NHEJ) repair mechanisms result in indel mutations in the genome caused by insertions and/or deletions[304]. Indels occurring within coding exons can result in frameshift mutations leading to premature stop codons or unstable truncated protein products.

Previous work has uncovered a novel role for FAK in controlling the composition of the immunosuppressive TME through FAK-dependent regulation of chemokines and cytokines[197]. Using a syngeneic transplantable cell model of SCC of the skin, FAK was found to translocate to the nucleus where it interacts with a number of transcription factors and transcriptional regulators to enhance the expression of a variety of chemokines and cytokines. This leads to elevated intra-tumoural Treg levels and suppression of the anti-tumour $CD8^+$ T-cell response. During the course of my PhD, more recent work published by the Denardo lab identified a role for FAK signalling within pancreatic cancer cells. They found FAK signalling from pancreatic cancer cells broadly regulates the fibrotic and immunosuppressive tumour microenvironment in favour of immunosuppression[142]. Specifically, they focused on FAK's regulation of tumour derived CXCL12 and its function in stromal expansion in PDAC. In order to further investigate how FAK regulates the immunosuppressive TME in pancreatic cancer, I chose to use of a syngeneic transplantable cell model of pancreatic cancer, the Panc47 cell line, isolated from pancreatic tumours arising on the KPC mouse model of PDAC. Using CRISPR/Cas9 genome editing I set out to deplete FAK expression and then reconstitute wild type FAK, thus creating two syngeneic cell lines that are genetically identical apart from FAK expression: FAK-wt and FAK-/-. This approach also allows investigation of the effects of any mutant FAK protein on an identical genetic background in PDAC.

In this chapter, I describe the target selection and design of sgRNA, construction of the CRISPR plasmid and the generation of a FAK-depleted cell line derived from LSL-$Kras^{G12D/+}$;LSL-$Trp53^{R172H/+}$ cell model of PDAC.

3.1.1 Aims

1) To generate a pancreatic cancer cell line model for the study of FAK function.

3.1.2 Experimental Approach

This chapter describes the use of CRISPR/Cas9 genome editing to deplete *fak* expression in a pancreatic cancer cell line, and the subsequent re-expression of wild-type FAK (FAK-wt) into selected FAK-depleted (FAK-/-) cell clones. **Figure 3.1** illustrates the experimental workflow followed. To investigate the role of FAK in regulating the immune environment in pancreatic cancer, the syngeneic transplantable cell model named T332047, hereafter referred to as Panc47, was utilised. Panc47 was originally derived from a PDAC tumour arising on the KPC mouse model of pancreatic cancer.

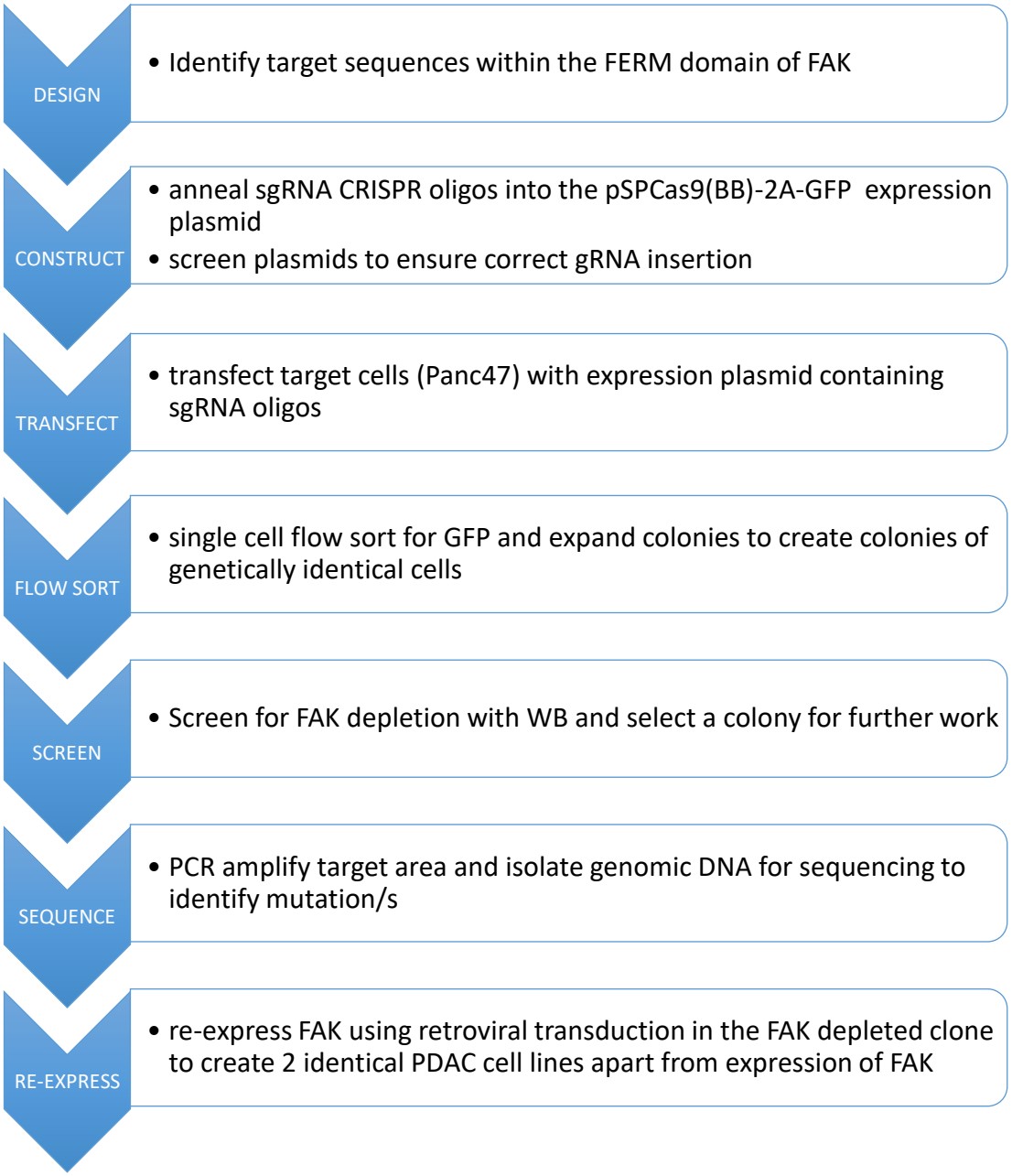


Figure 3.1 CRISPR/Cas9 experimental workflow

3.2 Results

3.2.1 Panc47 cells express FAK and nuclear FAK

FAK not only localises at sites of cell-extracellular matrix contact termed Focal Adhesions (FAs), but also translocates to the nucleus where the function remains poorly characterized[332]. Previous studies have established that nuclear FAK can regulate the expression of chemokines and cytokines in SCC cells, and that this plays a role in establishing an immuno-suppressive tumour environment required to evade the anti-tumour immune response in SCC[333]. We hypothesised that this could be a potential mechanism for immunosuppression in PDAC. Therefore I first set out to establish that FAK was expressed in Panc47 cells in order to ensure that this was a suitable model for studying FAK-dependent immune regulation in PDAC. Using whole cell lysates and biochemical fractionation to isolate cytoplasmic and nuclear extracts, followed by western blotting, protein extracts (nuclear, cytoplasmic and whole cell lysates) were probed for FAK **(figure 3.2)** and FAK was found to be readily detectable.

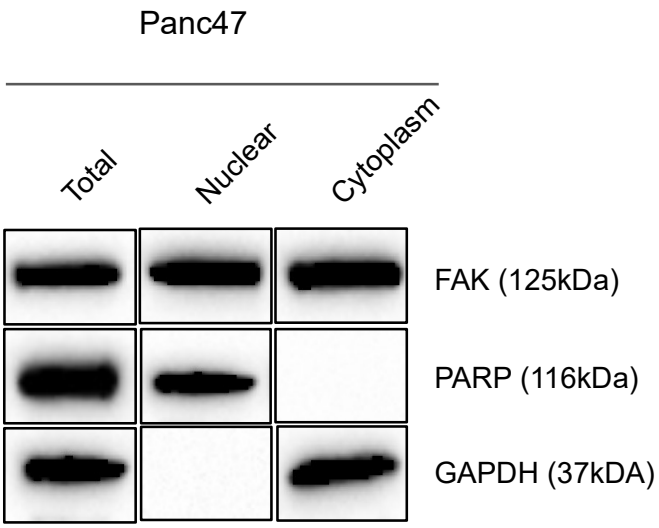


Figure 3.2 Parental PDAC cell line (Panc47) derived from LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$ pancreatic cancer cells express total and nuclear FAK.

Western blot of whole cell, nuclear and cytoplasmic fractions obtained after either cell lysis or nuclear fractionation of Panc47 cells. The western blot was probed with antibodies specific to FAK, PARP (nucleus) and GAPDH (cytoplasm), demonstrating efficient fractionation and the presence of cytoplasmic and nuclear FAK. Refer to appendix 1 for full western blot.

3.2.2 Guide sequence (sgRNA) design and plasmid construction.

Having identified that Panc47 cells express FAK and nuclear FAK, I used CRISPR/Cas9 genome editing to deplete FAK. The type II CRISPR-Cas system described in this thesis and in the protocol published by Ran et al [304] was derived from *S.pyogenes* bacteria. This system consists of two components: the Cas9 nuclease and a guide RNA (gRNA). The gRNA is comprised of the CRISPR RNA (crRNA) fused to the auxiliary trans-activating crRNA (tracrRNA). The tracrRNA is involved in initiating the cleavage process and the 20 nucleotides to the 5' end of the gRNA guides the Cas9 nuclease to the target gene of interest, in this case the FAK gene, through Watson-Crick base pairing[329]. For S.pyogenes derived Cas9 to function, the target cell DNA must immediately precede a 5'NGG-PAM (protospacer adjacent motif) sequence[329,330]. Although these targeting sites occur frequently at a rate of 1 in every 32bp[329], this must be taken into account when identifying appropriate gRNA sequences. The online CRISPR design tool (http://crispr.mit.edu/) was used to generate two pairs of complementary 20bp gRNAs (gFAK4 and gFAK6)

(**figure 3.3**). The gRNAs were designed to sites near the N terminus of the FAK gene, the region encompassing the FERM domain, in order to minimise the chances of a functional protein being produced following successful genome editing. Frameshift mutations are more likely to cause premature stop codons and therefore disruption in protein synthesis via nonsense mediated mRNA decay (NMD) if the stop codon is located near to the 5' end of a gene[334]. Given that the mutation is found in the N terminus region of the FERM domain, mutant mRNA transcripts would likely not be expressed or if expressed would be unstable, resulting in degradation. Additionally there is a single identified FAK promotor and therefore a single start site, thus making production of FAK isoforms not detected on WB unlikely. The CRISPR design software identifies suitable complementary pairs of gRNAs and then ranks them according to the number of potential off target binding sites, enabling selection of optimal sgRNA oligonucleotides. The resulting two top ranking guide oligonucleotide pairs (gFAK4 and gFAK6) were synthesised to include overhangs complementary to those created by Bbs1 digestion of the expression plasmid to allow annealing into the expression plasmid.

The mammalian expression plasmid pSPCas9(BB)-2A-GFP was chosen because it constitutively expresses the Cas9 protein and contains a GFP selection cassette to enable easy identification of cell clones where retroviral transfection into cells has been successful. Complementary guide oligonucleotides were annealed and cloned into this plasmid, and the plasmid transformed into chemically competent Top10 bacteria and cultured overnight in LB containing ampicillin to take advantage of selection using the ampicillin resistance gene within the plasmid. DNA was extracted from bacterial colonies and sent for sequencing using the U6 forward primer. Sequences were aligned to determine if they contained the inserted oligos; clones 1 of both gRNAs were positive **(figure 3.3)**.

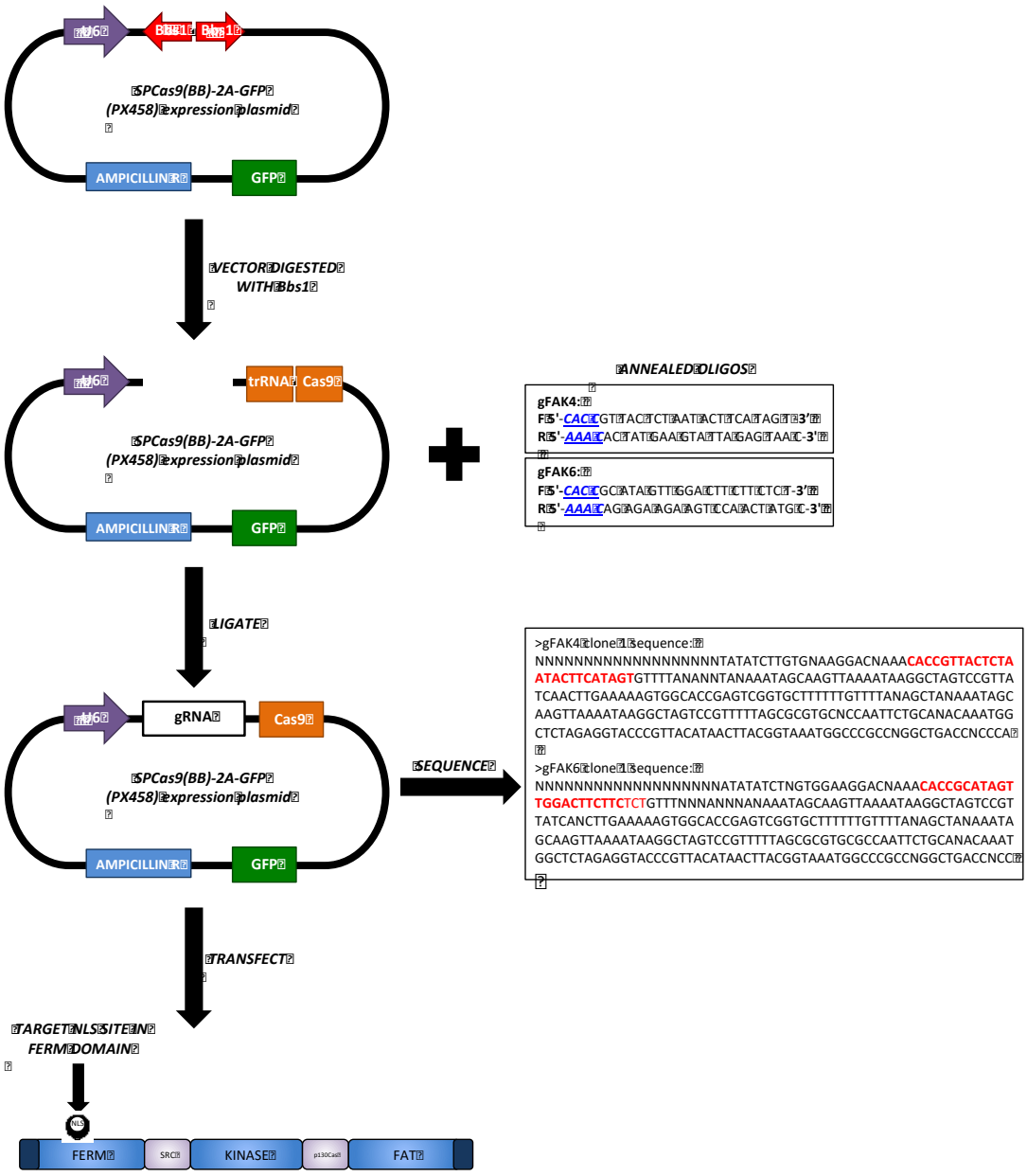


Figure 3.3 cloning strategy of guide sequence into pSPCas9(BB)-2A-GFP (PX458) expression plasmid.

Schematic of the pSPCas9(BB)-2A-GFP expression plasmid. The plasmid contains the ampicillin resistance gene (blue box), U6 promotor (purple arrow) and Cas9 expression cassette (orange box). Guide target sequences were synthesised as complementary oligonucleotides, annealed, and ligated into a BbsI cut expression plasmid upstream of tracrRNA scaffold to form the guide RNA (gRNA). Extra bases (blue italics) were added to 5'-ends of oligonucleotides to create matching overhangs to BbsI cut plasmid. Transcription of the gRNA was driven by the U6 promotor (purple arrow). The extract of sequence traces confirms successful cloning of CRISPR guide sequences into the expression plasmid. Guide sequences are highlighted in red. The plasmid was lipofectamine transfected into the target site of Panc47 at the NLS site of the F2 lobe of the FERM domain of FAK.

3.2.3 Clone generation and isolation

To generate FAK-depleted Panc47 cell clones, cells were transfected with the expression plasmids containing either the gFAK4 or gFAK6 guide sequences, and GFP positive clones were sorted into 96-well plates using FACS (Fluorescence-activated cell sorting). Resulting cell colonies were tested for successful depletion of FAK expression using anti-FAK western blotting. From a total of 16 colonies tested, 12 were identified to be FAK deficient (**figure3.4**).

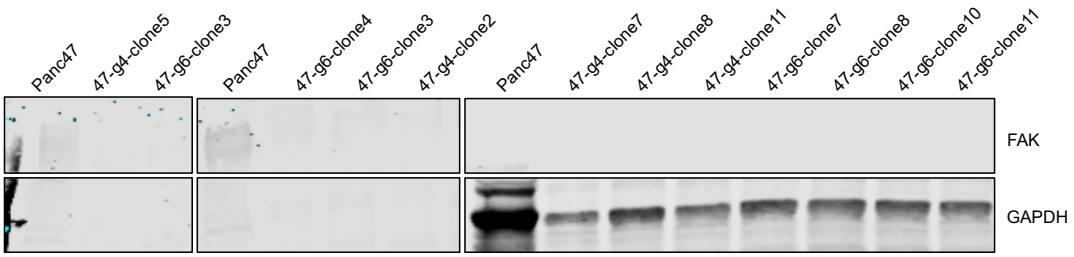


Figure 3.4 Screening of CRISPR clones derived from the parental Panc47 PDAC cell line

Western blots demonstrating successful knock out of clones: 47-g6-clone3, 47-g64clone2, 47-g4-clone7, 47-g4-clone8, 47-g4-clone11, 47-g6-clone7, 47-g6-clone11. Parental Panc47 is present as a positive control

3.2.4 Identification of the genetic mutation arising from CRISPR genome editing

One FAK depleted clone, 47-g4-clone2, was selected as it was a clear FAK deficient knock out on WB, displayed similar growth characteristics and appeared morphologically similar in cell culture to the other successful FAK knock out clones.. Therefore clone 47-g4-clone2 (hereafter referred to as FAK-/-) was chosen as a good representative clone for follow up work. Genomic cDNA was extracted and PCR amplified using FAK specific primers. Using the FAK forward and reverse primers again, direct sequencing of this PCR product was performed to confirm depletion of FAK and identify the mutation that had occurred (**figure 3.5**). Sequences were aligned using **ClutsalW (European Bioinformatics Institute)** software, identifying a 4 base pair deletion at position 579 of the cds (complete

coding sequence) (**figure 3.5A**). Comparison of the predicted protein sequence with that of wild-type FAK identified a premature STOP codon at amino acid position 193 (**figure 3.5B**) confirming that if a protein were to be produced, it would result in a severely truncated protein produced with a predicted molecular weight of approximately 22.4KDa. Western blotting using a FAK antibody raised to an epitope in the N-terminus did not detect a protein product of the predicted molecular weight, suggesting that if a product is produced then it is likely to be unstable and rapidly degraded (**figure 3.6A**).

Figure 3.5 Successful creation of CRISPR generated FAK -/- PDAC clone. Confirmation via genomic sequencing and protein alignment

A Sequencing of genomic region targeted by guide sequences showing the guide sequence and mutation created at position 579, aligned to sequence from mouse genome database. **B** Protein alignment demonstrating an early stop codon at AA position and a truncated protein

3.2.5 Reconstitution to create a FAK-wt clone

To generate a cell model with which to further study FAK function, I next re-expressed wild-type FAK (FAK-wt) into Panc47 FAK-/- cells using retroviral transduction. Successful re-expression of FAK-wt was confirmed using western blotting (**figure 3.6B**). Biochemical fractionation was used to isolate nuclear and cytoplasmic fractions. Western blotting of protein extracts was carried out using an anti-FAK antibody **(figure 3.6C)** and FAK was found to be readily detectable in the nucleus of Panc47 FAK-wt cells.

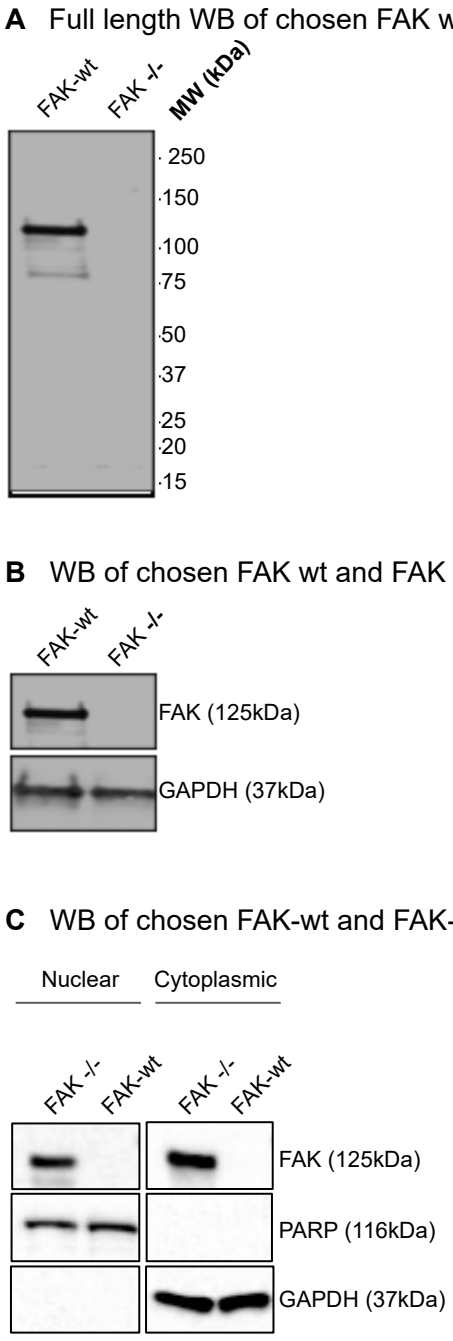


Figure 3.6 CRISPR generated FAK -/- PDAC clone and FAK-wt (reconstituted clone from 47-g4-clone2)

A Full length WB showing MW (molecular weight) markers (Bio-Rad Precision Plus Protein Dual Colour Standard), probed with monoclonal anti-FAK (4.47) antibody specific to the N-terminal domain of FAK, demonstrating absence of 22.4kDa fragment. **B** Cropped WB as in 3.6 A demonstrating absence of FAK in the CRISPR knock out cell line (FAK-/-) and additionally the re-expression in the FAK-wt cell line. **C** WB of nuclear and cytoplasmic fractions obtained after nuclear fractionation of FAK-/- and FAK-wt cell line. This demonstrates the absence of FAK and nuclear FAK in the FAK-/- cell line and presence of FAK and nuclear FAK in the FAK-wt cell line. The western blot was probed with antibodies specific to FAK, nuclear PARP and cytoplasmic GAPDH, demonstrating efficient fractionation and the presence of cytoplasmic and nuclear FAK.

3.3 Discussion

Using CRISPR genome editing, I have successfully generated a pancreatic cancer cell model that enables the study of cancer cell intrinsic FAK signalling in the regulation of pancreatic tumour growth and the immuno-suppressive TME. This approach was employed, as it is not only time efficient and cost-effective, but also because it facilitates detailed mechanistic downstream studies. An alternative approach would have been to generate a GEM model in which *fak* expression is specifically depleted in the pancreatic cancer cell of origin. This would have been possible through crossing mice carrying a conditional knockout floxed *fak* allele[335] with the KPC mouse to generate experimental *Pdx1 Cre LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$; FAK$^{flox/flox}$* mice. However, the drawbacks of this approach would have been the time taken to generate this model, in addition to some of the impracticalities involved in generating adequate KPC mouse numbers. Once this model is successfully created, not only is the mean latency approximately 4-5 months[310] for the KPC mice to generate pancreatic tumours, but KPC mice normally have to be enrolled in experiments on a rolling basis due the complicated breeding strategy and enrolment criteria[324]. This may have resulted in insufficient numbers to fill experimental groups. Therefore this approach would have been difficult to achieve in the time frame I had available to me. The use of implanted PDAC cell lines have been shown to have a similar histological appearance and immune cell profile to those PDACs arising spontaneously in the KPC mouse model[324] and therefore a transplantable model was deemed the most suitable model system to use.

CRISPR/Cas 9 gene editing technology was employed to create a FAK knock out line in the Panc47 cells. This is an efficient and reliable gene-editing tool and has largely superseded other nuclease gene editing technologies, thus was the most appropriate choice. In this chapter I show successful FAK-depletion using CRISPR gene editing in 12 clones. One clone was carried forward for further experiments and FAK re-expression. There are however some drawbacks of this technology that can affect specificity and efficiency. Two potential concerns with use of plasmid transfection is the possibility for immune responses to viral particles[336] and long lasting Cas9:sgRNA expression, increasing the potential for off target genome editing after it has successfully modified the target locus[337]. Since commencing my

PhD studies there have been some advances in CRISPR/Cas9 genome editing technology, developed to overcome some of these issues. Direct delivery of Cas9:sgRNA ribonucleotide protein complexes (RNPs) into cells via cationic lipid delivery[338,339] and cell penetrating peptide-mediated delivery[336,340] can now be achieved to allow for transient Cas9 activity without the use of viral transfection. Systems inducing temporal Cas9 activity through light activation[341] or small molecule activation[342,343] can also be employed to address concern around the longevity of Cas9 activity within cells. These new technologies have been shown to not only increase the efficiency but also the specificity of genome modification compared to transfection techniques.

In this chapter I describe the successful generation of a FAK-depleted pancreatic cell line and re-expression of FAK-wt to create a genetically identical cell line, other than expression of FAK, to allow dissection of FAK's contribution to the immunosuppressive TME present in PDAC. The comparison of these two syngeneic clones allows clinically relevant insights into the immunotherapeutic potential of pharmacological inhibitors of FAK's function in cancer treatment.

4 The role of FAK in tumour growth and on the tumour myeloid population *in-vivo*

4.1 Introduction

Previous studies using mouse models of pancreatic cancer have demonstrated that treatment with FAK kinase inhibitors can impair pancreatic tumour growth[142,303]. However, the precise mechanisms that underpin this response have not been fully elucidated. While FAK is known to regulate multiple cellular processes that can impact on tumour growth, including cancer cell cycle progression, adhesion, polarisation, migration, apoptosis, proliferation and survival[263], emerging evidence has also identified a role for FAK in regulating the pancreatic tumour microenvironment. For example, inhibition of pancreatic tumour growth in response to treatment with the FAK inhibitor PF562271 was associated with a reduction in tumour infiltrating macrophages. In support of this, Jiang et al identified that treatment with the pharmacological inhibitor VS4718 resulted in reduced growth of pancreatic tumours and this was associated with broad reprogramming of the immune environment, including macrophage regulation[142]. This study, published during the course of my PhD, used short hairpin RNA (shRNA) to deplete FAK expression in KPC-derived pancreatic cancer cell lines and demonstrated reduced tumour growth in response to FAK depletion. Thus, FAK regulates pancreatic tumour growth and the immunosuppressive tumour environment. However, the mechanisms that govern this remain poorly characterised.

Components of the PDAC TME are known to promote tumour growth and progression. The highly immunosuppressive TME is characteristic of PDAC and stromal components occupy the majority of the tumour mass[39–41]. Many of these cell types have been shown to promote pancreatic tumour growth. These include macrophages, MDSCs, Tregs, T_H2 cells, CAFs, myofibroblasts and endothelial cells[48,49]. The most numerous immunosuppressive population is the extensive myeloid cell population, with tumour associated macrophages (TAMs) being the most abundant, far outnumbering other immunosuppressive cells types such as the more scarce T-cell population[56,64]. The high numbers of TAMs in the PDAC TME perhaps reflects their critical role in pancreatic tumour progression. TAMs exhibit

extensive heterogeneity in function and phenotype, and grouping these different subpopulations is a constantly evolving topic. Macrophages historically have been subdivided into 'classically activated' macrophages and 'alternatively activated' macrophages, now designated as M1 and M2 macrophages respectively[344,345]. M2 macrophages are gaining increasing interest due to their correlation with a poorer prognosis and ability to modify and maintain the immunosuppressive TME in various tumour types[64]. In a study analysing surgical patients with pancreatic cancer, accumulation of M2 TAMs correlated with earlier metastasis, larger tumour size and shorter survival times[65]. Traditionally it was also thought that TAMs were derived from circulating monocytes, but recent evidence suggest that in the normal pancreas, tissue resident macrophages are almost entirely embryonically derived from the yolk sac[346], and that both resident and recruited Ly6C^{hi} bone marrow derived macrophages are expanded through in situ proliferation during pancreatic tumour progression[56]. It is likely that phenotypically distinct macrophage subsets possessing different functions exist in PDAC. The mechanism and complexity of the FAK driven TAM compartment within the PDAC TME and the expression pattern that clearly define the macrophage subsets regulating immunosuppression in PDAC models is yet to be fully unravelled[56,347]. Therefore I focused on initially exploring FAK-dependent regulation of the tumour myeloid compartment using orthotopic implantation of the two syngeneic PDAC cell lines: Panc47 FAK-wt and Panc47 FAK-/-, derived from Panc47 *LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$,* the generation of which is described in chapter 3. These cell lines are genetically identical apart from FAK expression and therefore this approach allows for investigation of the effects of mutant FAK *in-vivo* on an identical genetic background.

4.1.1 Aims

1. To determine whether FAK-depletion in Panc47 cells results in a tumour growth delay and improved survival.
2. To determine whether FAK regulates the immune cell infiltrate in orthotopic Panc47 tumours.

4.2 Results

4.2.1 FAK-depletion delays tumour growth, resulting in improved overall survival

FAK inhibition has previously been shown to impair pancreatic tumour growth[142,303]. I therefore set out to identify whether FAK depletion in Panc47 cells resulted in a tumour growth delay and improved overall survival. $0.5x10^6$ Panc47 FAK-wt or FAK-/- cells were implanted into the pancreas of immune competent C57BL/6 mice under sterile surgical conditions and allowed to grow for either two or three weeks, before harvesting and weighing the tumours. Tumours were analysed by a pathologist. FAK-wt and FAK-/- tumours were largely comparable (**table 4.1**) but the FAK-wt tumours had a more fibrotic stroma, consistent with other studies reported in the literature with FAK depletion in PDAC[142]. Implanted tumours were also largely comparable to tumours arising in the KPC GEM model of pancreatic adenocarcinoma. Average tumour weight was calculated and plotted as a line graph of tumour weight over time (**figure 4.1 A**). Results show a significant reduction in tumour weight at both 2 and 3 weeks in the Panc47 FAK-/- tumours when compared with Panc47 FAK-wt tumours, consistent with previous reports[142,303]. To determine whether this tumour growth delay resulted in an improvement in overall survival, 0.5×10^6 Panc47 FAK-wt or FAK-/- cells were implanted into the pancreas of C57BL/6 mice, and mice were monitored for terminal symptoms caused by pancreatic tumour growth including weight loss equal or exceeding 10% of starting weight, signs of abdominal pain and abdominal distension. If any one symptom became apparent, the animal was euthanized. The results show a small but significant survival benefit of FAK depletion (**figure 4.1 B**).

	KPC GEM			FAK-wt		FAK-/-	
TUMOUR ID	nKPC 82.5g	nKPC 102.2b	nKPC 104.1a	FAK-wta	FAK-wtb	FAK-/-a	FAK-/-b
MITOTIC RATE	3	3	1	3	5	4	3
FIBROUS STROMA	Moderate	Marked	Marked	Moderate	Moderate	Mild	Mild
NECROSIS	Mild	Moderate	Marked	Mild	Mild	Mild	Mild
INFILTRATING LEUKOCYTES	Mild	Mild	Moderate	Mild	Mild	Mild	Mild
PREDOMINANT LEUKOCYTE	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)	Granulocytes (neutrophils/ G-MDSCs)
TUMOUR INFILTRATION	Marked	Marked	Marked	Moderate	Marked	Marked	Moderate
ANAPLASIA	Moderate	Marked	Mild	Mild	Mild	Mild	Mild

Table 4.1 Histological comparison tumours arising on the KPC model of pancreatic adenocarcinoma and implanted FAK-wt and FAK-/- tumours

Histological analysis for table courtesy of Dr James Baily, *BVM&S MSc PhD DiplECVP MRCVS*

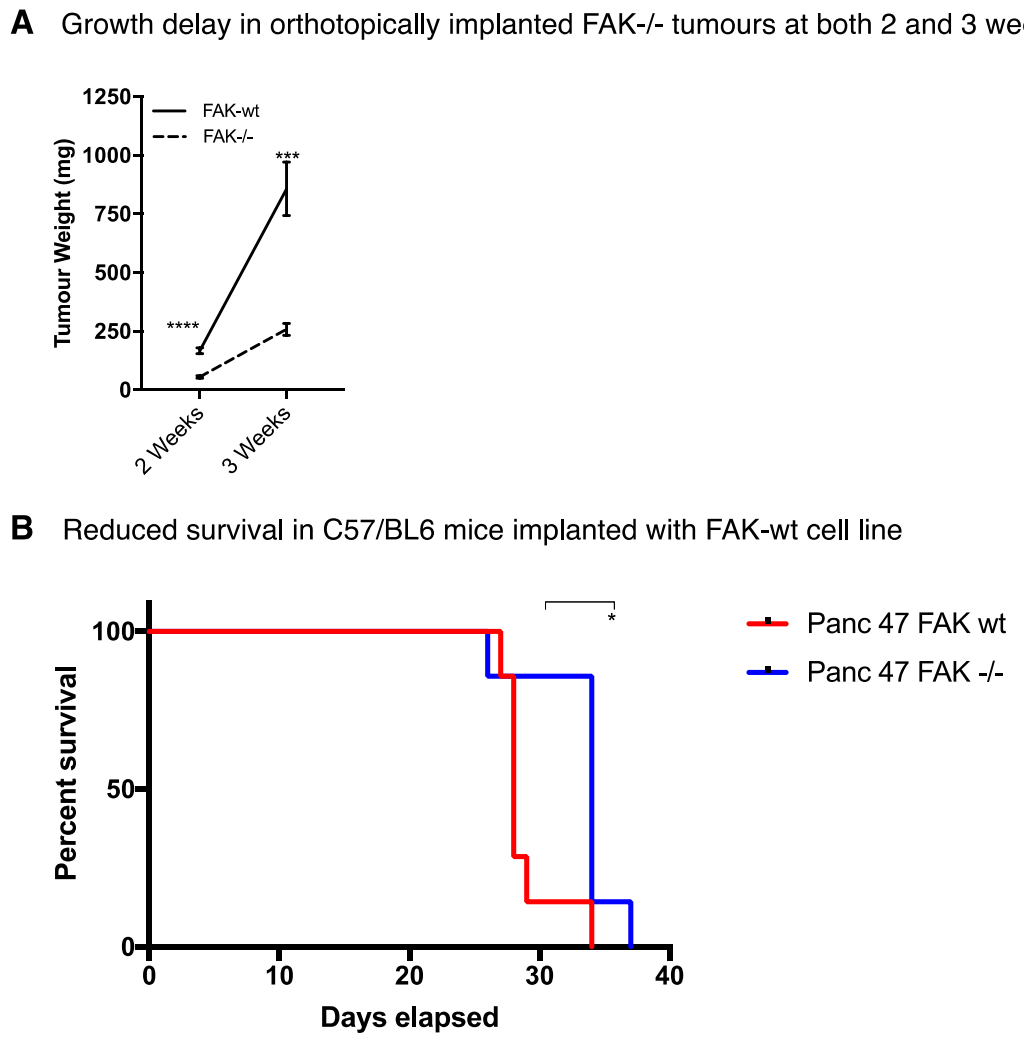


Figure 4.1 FAK-depletion in Panc47 pancreatic cancer cells results in impaired tumour growth and improved overall survival

The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK wt or FAK -/- cell lines **A** Tumours were harvested at two or three weeks (n= 20 mice per group; error bars represent SEM; statistical test used was Students T-test). **B** Survival curves of mice with pancreatic implantation of Panc47 FAK-wt or FAK-/- cell lines. Mice were sacrificed once they presented with terminal symptoms related to pancreatic neoplasia (n= 7 mice per group; error bars represent SEM; statistical test used was Log-rank). FAK-depletion in Panc47 pancreatic cancer cells results in impaired tumour growth and reduced overall survival. P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

4.2.2 CD8$^+$ T-cell depletion restores FAK-dependent growth delay

Previous studies have demonstrated a link between FAK expression and regulation of immunosuppressive cell types that then inhibit cytotoxic T-cells within the TME in murine models of SCC[301] and pancreatic cancer[142]. I therefore sought to determine whether the observed growth delay was a consequence of an anti-tumour

immune response. To investigate this, C57BL/6 mice were treated with a $CD8^+$ T-cell depleting antibody and 0.5 x 10^6 Panc47 FAK-wt or FAK-/- cells were implanted into the pancreas. $CD8^+$ T-cell depletion was initiated prior to tumour implantation to achieve a $CD8^+$ T-cell depleted background to ensure these cells did not play a role in the biology of tumour progression. Depletion was maintained throughout the course of the experiment by a twice-weekly injection of anti-CD8 antibody (**figure 4.2 A**). Mice were sacrificed two weeks after tumour cell implantation and tumours weighed. Tumour growth was significantly restored in anti-CD8 treated mice bearing Panc47 FAK-/- tumours (**figure 4.2 B**) when compared with isotype control antibody treated mice. No effect on tumour growth was evident between isotype control and anti-CD8 antibody treated mice bearing Panc47 FAK-wt tumours. These results imply that a significant proportion of the Panc47 FAK-/- tumour growth delay is the result of an anti-tumour $CD8^+$ T cell response.

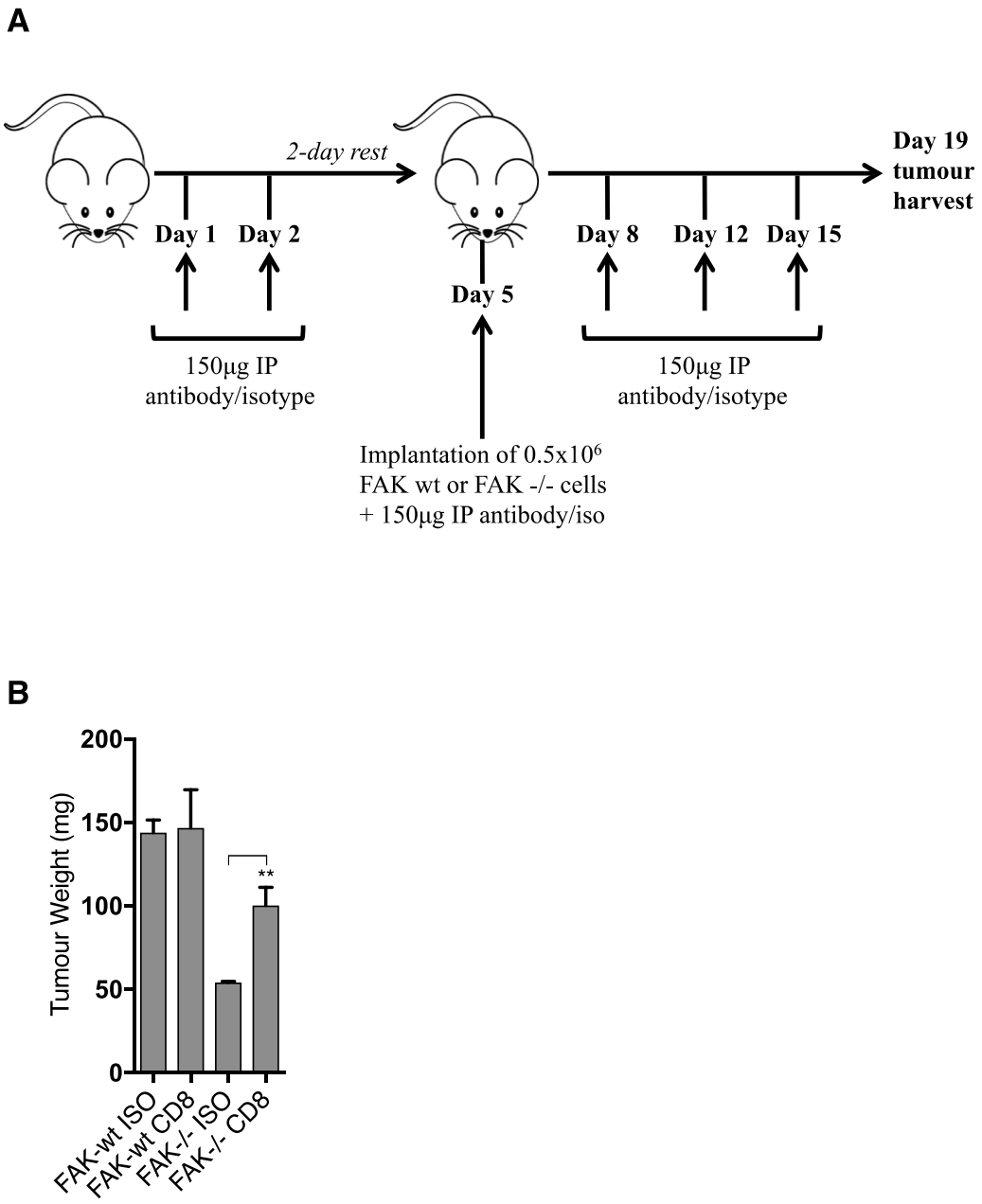


Figure 4.2 Pancreatic tumour growth +/- CD8$^+$ T-cell depletion and FAK-depletion.

The pancreas of C57BL/6 mice were implanted with 0.5x10^6 Panc47 FAK-wt or FAK-/- cell lines. Groups (n= 6 per group) were treated with either CD8$^+$ depleting antibody or isotype control. **A** Dosing schedule **B** Tumours were harvested and weights recorded at 14 days post implantation (n= 6 per group; error bars represent SEM; statistical test used was Students T-test, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

4.2.3 FAK promotes M2 polarisation of Ly6C$^+$ TAMs

Recruitment and expansion of immune cells including macrophages, MDSCs and Tregs with intrinsic immunosuppressive capabilities have been shown to suppress

the anti-tumour immune response and promote tumour survival in pancreatic cancer[48]. Therefore, I next sought to determine whether FAK driven changes in these immunosuppressive populations could correlate with the $CD8^+$ T-cell mediated change in the observed growth characteristics between Panc47 FAK-wt and FAK-/- tumours. Thus I set out to investigate whether cancer cell FAK signalling influences changes in the abundance or phenotype of these immune populations. $0.5x10^6$ Panc47 FAK-wt or FAK-/- cells were implanted into the pancreas of C57BL/6 mice and tumours harvested at two weeks for disaggregation and analysis by flow cytometry with stain 1 and stain 2 (**table 4.2**). The immune cell markers and gating strategy used to identify Tregs and myeloid populations are demonstrated in **table 4.3** and **figure 4.3 A** and **B** respectively. Immune profiling of Panc47 FAK-wt and FAK-/- tumours identified that FAK depletion results in a significant reduction in Tregs known to influence cytotoxic T-cell activity (**figure 4.4**).

Detailed analysis identified four distinct populations of macrophages within pancreatic tumours defined by their expression level of CD11b and Ly6C: $CD45^+CD11b^{hi}F480^+Ly6C^+$ (inflammatory macrophage/ TAM), $CD45^+CD11b^{hi}F480^+Ly6C^-$ (resident macrophage), $CD45^+CD11b^{lo}F480^+Ly6C^+$ and $CD45^+CD11b^{lo}F480^+Ly6C^-$ macrophages. Recent evidence has shown differential expression of CD11b and Ly6C in pancreatic TAMs based on whether these macrophages were derived from tissue resident or bone marrow recruited macrophages[56], however further analysis was not carried out to determine which population these cells were derived from. The total frequency of macrophages was not found to be different between Panc47 FAK-wt and FAK-/- tumours, and neither was the frequency of the different macrophage subsets. Total levels of G-MDSC and M-MDSCs were also not different (**figure 4.4**). Using markers indicative of classical M1 macrophage activation (MMR^-CD86^+) and the alternative M2 activation (MMR^+CD86^-), I found a shift in the proportion of alternatively activated M2-like polarised macrophages within the subpopulations of macrophages identified when comparing Panc47 FAK-wt and FAK-/- tumours. The proportion of alternatively activated M2-like polarised macrophages was significantly higher in the $Ly6C^+$ subpopulations ($CD45^+CD11b^{hi}F480^+Ly6C^+$ and

$CD45^{+}CD11b^{lo}F480^{+}Ly6C^{+}$) within Panc47 FAK-wt tumours when compared to Panc47 FAK-/- tumours. The proportions of classically activated M1-like macrophages in these same macrophage subpopulations were significantly higher in Panc47 FAK-/- tumours verses Panc47 FAK-wt tumours (**figure 4.5**). This suggests that FAK is polarising the $Ly6C^{+}$ macrophage subpopulations to an M2-like TAM phenotype.

STAIN 1	
MARKER	**FLUOROPHORE**
Ly6G	BUV395
CD140a	BV650
F480	BV421
CD11b	BV510
CD31	BV605
PD-L1/CD274	BV711
Ly6C	PerCP/Cy5.5
MMR	PE
CD11c	PE/DAZZLE
CD86	PE/Cy7
PD-L2/CD273	APC
CD45	AF700
Viability	Zombie NIR

STAIN 2	
MARKER	**FLUOROPHORE**
CD4	BV510
CD25	BV650
FoxP3	AF647
CD45	AF700
Viability	Zombie NIR

Table 4.2 Flow cytometry stain used to identify myeloid (stain 1) and Treg (stain 2) populations in PDAC tumours

Population	Markers
Macrophage	$CD45^{+}CD11b^{hi/lo}$ $F480^{+}$
Inflammatory Macrophage/ tumour Associated Macrophage	$CD45^{+}CD11b^{+}$ $F480^{+}$ $Ly6C^{+}$
Resident Macrophage	$CD45^{+}CD11b^{+}$ $F480^{+}$ $Ly6C^{-}$
Inflammatory Macrophage/ TAM (M2 polarisation)	$CD45^{+}CD11b^{hi/lo}$ $F480^{+}$ $Ly6C^{+}$ MMR^{+} $CD86^{-}$
Inflammatory Macrophage/ TAM (M1 polarisation)	$CD45^{+}CD11b^{hi/lo}$ $F480^{+}$ $Ly6C^{+}$ MMR^{-} $CD86^{+}$
Resident Macrophage (M2 polarisation)	$CD45^{+}CD11b^{hi/lo}$ $F480^{+}$ $Ly6C^{-}$ MMR^{+} $CD86^{-}$
Resident Macrophage (M1 polarisation)	$CD45^{+}CD11b^{hi/lo}$ $F480^{+}$ $Ly6C^{-}$ MMR^{-} $CD86^{+}$
Granulocytic Myeloid Derived Suppressor cell (G-MDSC)	$CD45^{+}CD11b^{+}$ $F480^{-}$ $Ly6C^{lo}$ $Ly6G^{+}$
Monocytic Myeloid Derived Suppressor cell (M-MDSC)	$CD45^{+}CD11b^{+}$ $F480^{-}$ $Ly6C^{hi}$ $Ly6G^{-}$
Endothelial cells	$CD31^{+}CD45^{-}$
Dendritic cells ($CD11b^{-}$)	$CD45^{+}CD11b^{-}CD11c^{+}$
Dendritic cells ($CD11b^{+}$)	$CD45^{+}CD11b^{+}$ $F480^{-}$ $Ly6G^{-}Ly6C^{-}$ $CD11c^{+}$
T cells/ B cells	$CD45^{+}CD11b^{-}CD11c^{-}Ly6G^{-}Ly6C^{-}$
Tumour cells/ fibroblasts	$CD31^{-}CD45^{-}$
Regulatory T cell (Treg)	$CD45^{+}$ $CD3^{+}$ $CD4^{+}$ $FoxP3^{+}$ $CD25^{+/-}$

Table 4.3 Cells within the PDAC TME and the markers used to identify them by flow cytometry

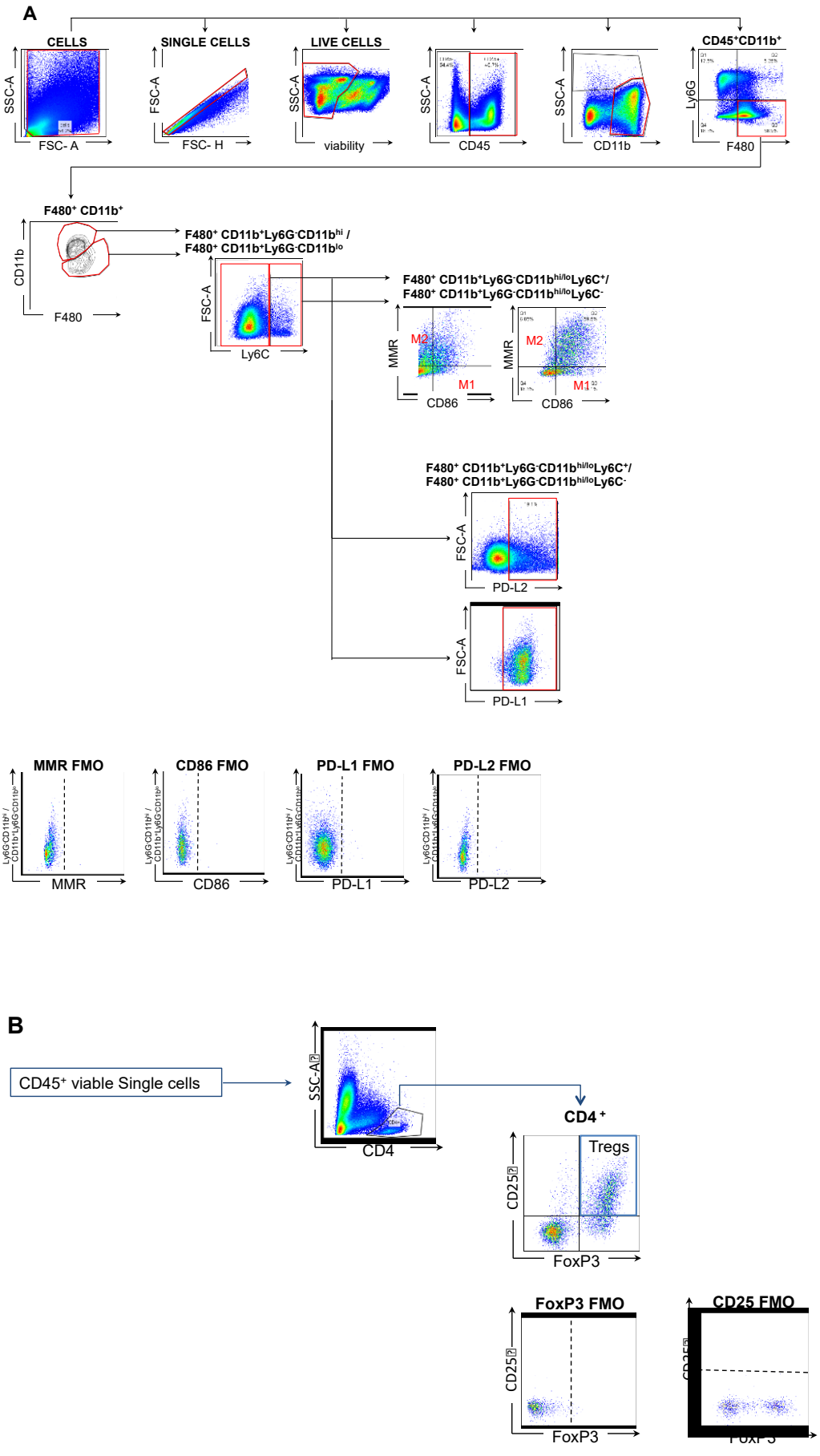

A
CELLS
SSC-A
FSC- A
SINGLE CELLS
FSC-A
FSC- H
LIVE CELLS
SSC-A
viability
SSC-A
CD45
SSC-A
CD11b
CD45+CD11b+
Ly6G
F480
F480+ CD11b+
CD11b
F480
F480+ CD11b+Ly6G-CD11bhi /
F480+ CD11b+Ly6G-CD11blo
FSC-A
Ly6C
F480+ CD11b+Ly6G-CD11bhi/loLy6C+/
F480+ CD11b+Ly6G-CD11bhi/loLy6C-
MMR
M2
M1
CD86
MMR
M2
M1
CD86
F480+ CD11b+Ly6G-CD11bhi/loLy6C+/
F480+ CD11b+Ly6G-CD11bhi/loLy6C-
FSC-A
PD-L2
FSC-A
PD-L1
MMR FMO
MMR
CD86 FMO
CD86
PD-L1 FMO
PD-L1
PD-L2 FMO
PD-L2
B
CD45+ viable Single cells
SSC-A
CD4
CD4+
Tregs
CD25
FoxP3
FoxP3 FMO
CD25
FoxP3
CD25 FMO
CD25
FoxP3

Figure 4.3 Gating strategy for flow cytometry analysis of Tregs, tumour macrophages and their polarisation status.

A Gating strategy applied to stain 1 (Table 4.1) and FMO control samples to determine proportions of TAMs. TAM polarisation was determined using MMR and CD86 expression. **B** Gating strategy applied to stain 2 (Table 4.1) to identify Tregs and FMO control samples used to determine Treg population. Each FMO control sample contains every marker in stain 2 except for the named marker. FMO control samples were used to determine the correct gating positions in flow cytometry analysis to exclude false positive results. FMO=Fluorescence minus one, FSC-A= forward scatter area, SSC-A= side scatter area.

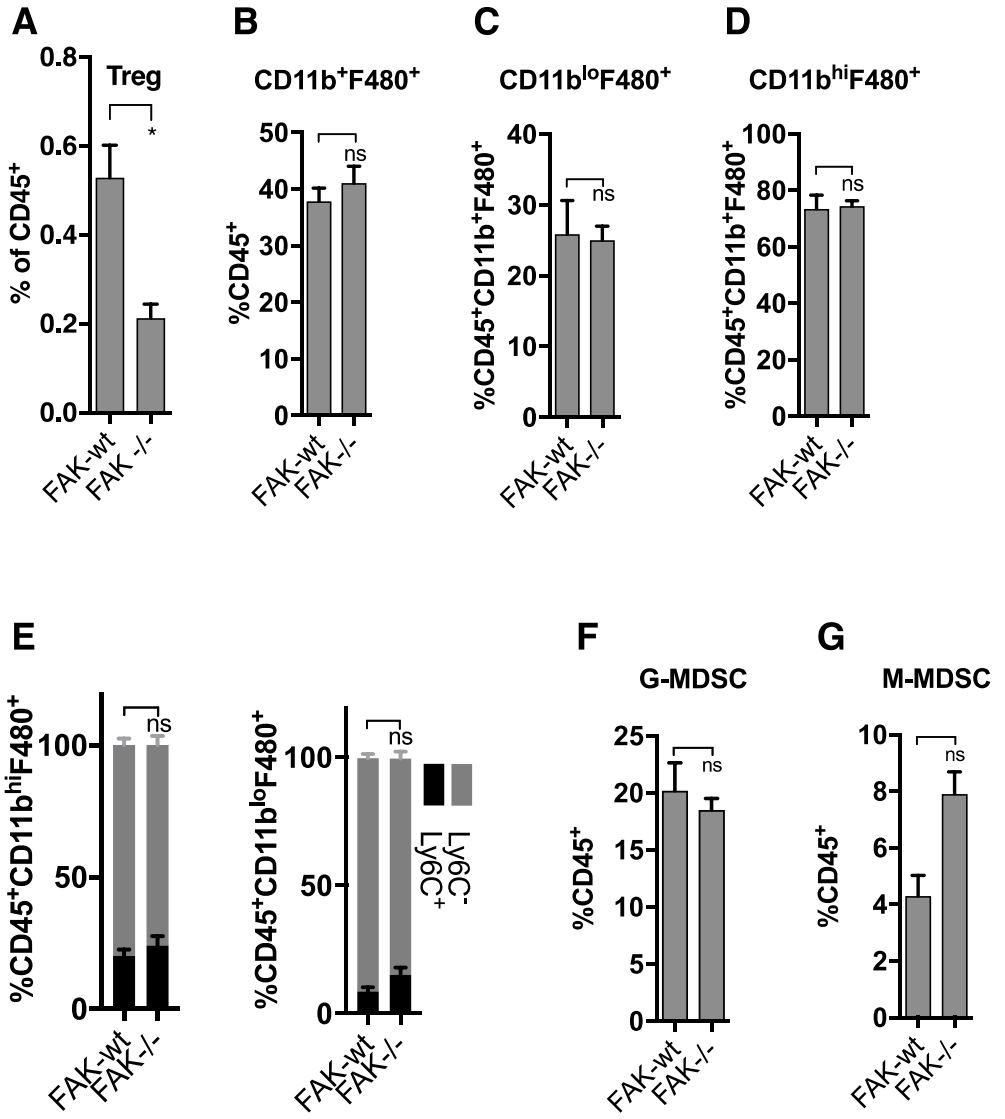


Figure 4.4 FAK-depletion in Panc47 pancreatic cancer cells alters total Treg levels but does not alter total levels of intra-tumoural macrophage, G-MDSC or M-MDSC.

The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt or FAK -/- cell lines and harvested at 14 days post implantation. (n= 3 per group; error bars represent SEM; statistical test used was Students T-test), gating and stains as described in figures 4.3 and table 4.1 respectively. **A** total intra-tumoural Tregs, **B** total intra-tumoural macrophages **C** $CD11b^{lo}$ intra-tumoural macrophages **D** $CD11b^{hi}$ intra-tumoural macrophages **E** $CD11b^{hi/lo}$ $Ly6C^{+/-}$ intra-tumoural macrophages **F** G-MDSCs and **G** M-MDSCs

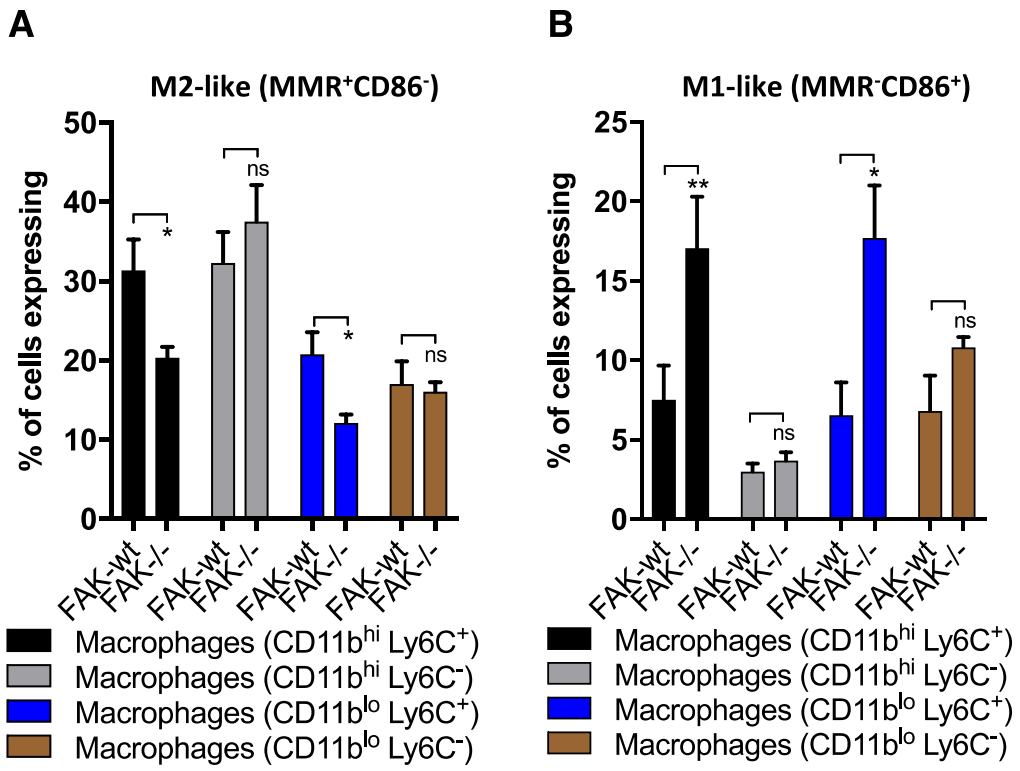


Figure 4.5 FAK-depletion in Panc47 pancreatic cancer cells results in M2-like to M1-like polarisation of Ly6C$^+$ tumour associated macrophages

The pancreas of C57BL/6 mice were implanted with 0.5x10^6 Panc47 FAK-wt or FAK -/- cell lines and harvested at 14 days post implantation. (n= 3 mice per group; error bars represent SEM; statistical test used was Students T-test, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.2) **A** M2 (CD86$^+$ MMR$^-$) in CD11b$^{hi/lo}$Ly6C$^{+/-}$ macrophages in FAK-wt and FAK-/- pancreatic tumours **B** M1 (CD86$^-$ MMR$^+$) in CD11b$^{hi/lo}$Ly6C$^{+/-}$ macrophages in FAK-wt and FAK-/- pancreatic tumours

4.2.4 FAK promotes PD-L2 expression in the myeloid cell compartment

PD-L1 (also known as B7-H1/ CD274) is widely expressed whereas PD-L2 (also known as B7-DC/ CD273) has a more restricted expression pattern and both are ligands for PD-1, a co-inhibitory receptor expressed by activated T-cells that acts as an immune checkpoint to regulate T-cell activity. Both ligands have been found to be over expressed in post-surgical PDAC specimens[258] and are thought to be

involved in immune evasion in cancer. Therefore, I sought to investigate whether expression of PD-L1 and / or PD-L2 was different in myeloid cell subsets present in Panc47 FAK-wt and FAK-/- tumours. $0.5x10^6$ Panc47 FAK-wt or FAK-/- cells were implanted into the pancreas of C57BL/6 mice and tumours harvested at two weeks for disaggregation and analysis by flow cytometry with stain 1 (**table 4.2**). The immune cell markers and gating strategy used to identify the various myeloid populations and macrophage subpopulations are demonstrated in **table 4.3** and **figure 4.2** respectively. Both macrophages and MDSCs from Panc47 FAK-/- tumours were found to have reduced surface expression of the immune checkpoint ligand PD-L2 but not PD-L1 (**figure 4.6**). PD-L1 expression was reduced on Panc47 FAK-/- tumour cells/ stromal fibroblasts. This is an interesting finding but was not investigated further in this study. PD-L2, induced on monocytes and macrophages by CSF1, IL4, and INF-γ [348], is linked to immunosuppressive macrophage function[349]. I demonstrate that FAK expression in pancreatic cancer cells increases the percentage of macrophages expressing PD-L2, implying that FAK-dependent signalling in pancreatic cancer cells can influence the immunosuppressive function of TAMs. PD-L2 has a more restricted expression profile than PD-L1, but expression is not restricted to the macrophage population and has also been reported on dendritic cells[350],endothelial cells[351], tumour cells[352,353],B-cells[354], T_H2 cells[355] and MDSCs[356]. I therefore looked at PD-L2 expression on other cells in the PDAC TME and identified regulation of PD-L2 expression in the wider myeloid population including G-MDSCs and M-MDSCs (**figure 4.7 A**). PD-L1 expression was not regulated (**figure 4.7 B**).

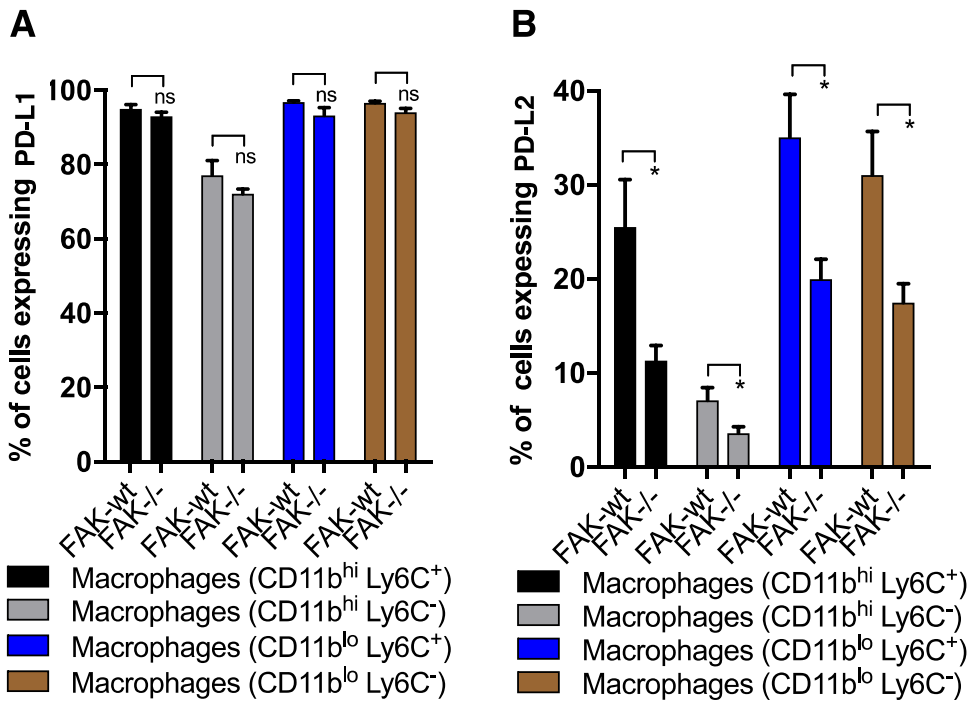


Figure 4.6 FAK-depletion in Panc47 pancreatic cancer cells results in reduced surface expression of the immune checkpoint ligand PD-L2 but not PD-L1 in all tumour associated macrophage subsets

The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt or FAK-/- cell lines and harvested at 14 days post implantation. (n= 3 per group; error bars represent SEM; statistical test used was Students T-test, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.2 **A** PD-L1 expression on $CD45^+CD11b^{hi/lo}F480^+Ly6C^{+/-}$ TAM **B** PD-L2 expression on $CD45^+CD11b^{hi/lo}F480^+Ly6C^{+/-}$ TAM

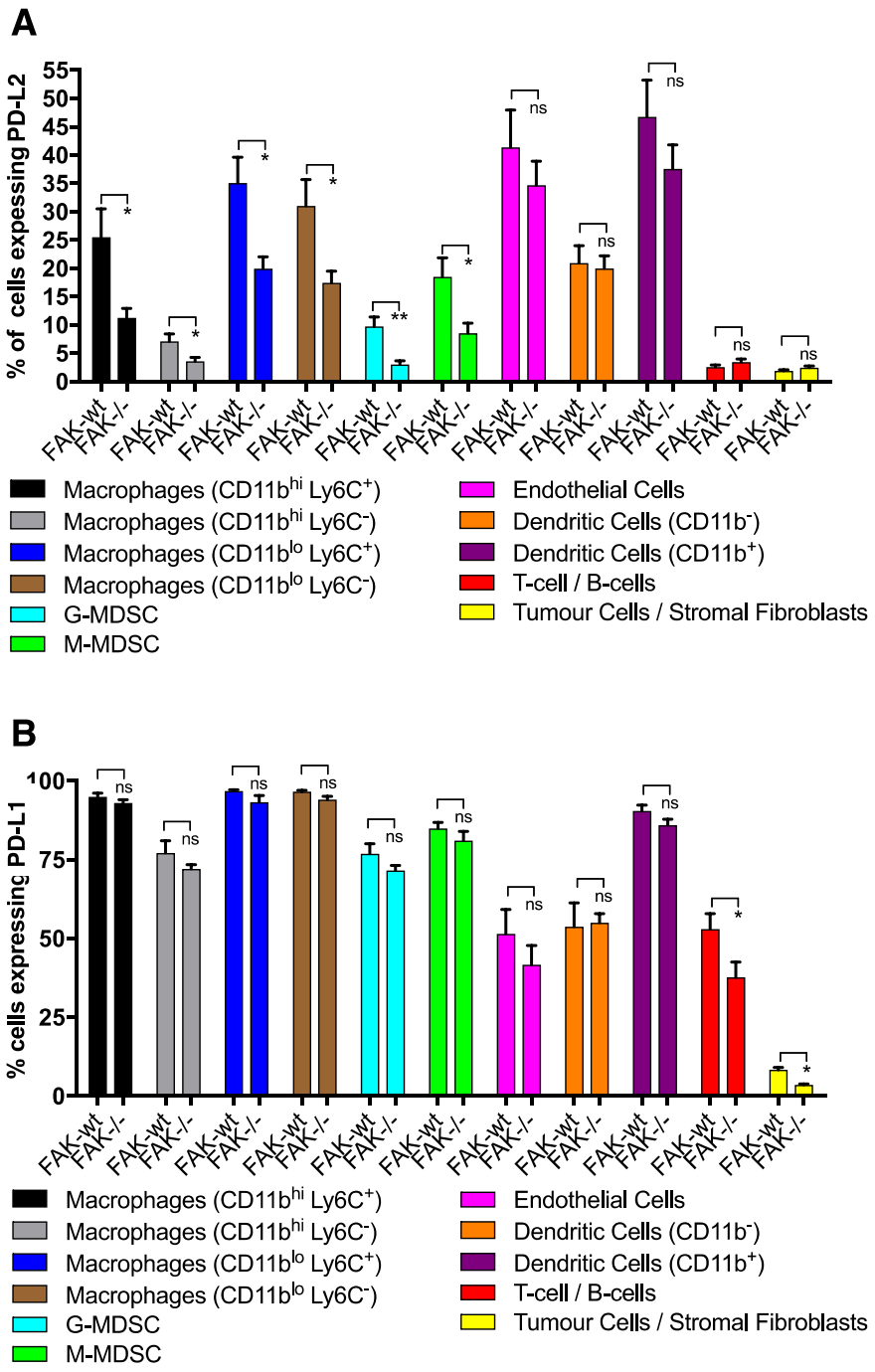


Figure 4.7 FAK-depletion in Panc47 pancreatic cancer cells results in reduced surface expression of the immune checkpoint ligand PD-L2 specifically in the myeloid compartment

The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt or FAK-/- cell lines and harvested at 14 days post implantation. (n= 3 per group; error bars represent SEM; statistical test used was Students T-test, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.2 and immune cell population markers as described in table 4.1. **A** PD-L2 expression on $CD45^+CD11b^{hi/lo}F480^+Ly6C^{+/-}$ TAM, G-MDSCs, M-MDSCs, endothelial cells, dendritic ($CD11b^{-/+}$) cells, T/B cells and other cells (presumed to be tumour cells and stromal fibroblasts). **B** PD-L1 expression on $CD45^+CD11b^{hi/lo}F480^+Ly6C^{+/-}$ TAM, G-MDSCs, M-MDSCs, endothelial cells, dendritic ($CD11b^{-/+}$) cells, T/B cells and other cells (presumed to be tumour cells and stromal fibroblasts).

4.3 Discussion

In this chapter I show that cancer cell intrinsic FAK signalling regulates Panc47 tumour growth and overall survival, at least in part through regulating the anti-tumour $CD8^+$ T-cell response. This mirrors clinical findings in surgical cases where increased levels of FAK expression correlate positively with pancreatic tumour size[300] and negatively with patient survival[142].

For the purpose of this study I elected to implant the pancreatic tumour cell lines into the pancreas of immune competent mice. My main interest is the immune TME and therefore this method was selected in order to recapitulate the local immune environment as closely as possible. Tumour growth was estimated by the difference in tumour weight at 2 and 3 weeks. The pitfall of this method is lack of kinetic measurements over time. Growth of tumours implanted subcutaneously can be more easily monitored using callipers, however orthotopically implanted tumours, although more difficult to monitor due to their intra-abdominal position, have the advantage of developing in their native organ. One way to overcome the difficulty in monitoring intra-abdominal tumour growth in live mice is through sequential MRI scans[303], ultrasound imaging[324] or through the use of bioluminescence imaging as PDAC cell lines can be engineered to express a luciferase reporter[324]. MRI has significant cost implications due to equipment requirements and therefore would likely not be the method of choice for my study but all of these methods would allow for longitudinal imaging of tumour growth, thus refining my experimental approach.

FAK has a number of cellular functions in cancer including regulation of cancer cell proliferation, survival, invasion, migration and adhesion[267]. There are consequently a multitude of FAK functions that could account for an increase in tumour growth and reduced mouse survival in this model. Previous work has demonstrated a novel function for FAK in mediating SCC growth in immune competent FVB mice via FAK-dependent regulation of chemokines and cytokines. These changes in the chemokine/cytokine and ligand-receptor networks were found to control the composition of the immunosuppressive TME to increase numbers of

immunosuppressive Tregs and inhibit antigen-primed cytotoxic $CD8^+$ T cell activity, permitting growth of FAK-expressing SCC tumours. I therefore wanted to explore the possibility of FAK dependent changes in the pancreatic tumour TME, and whether these changes contribute to the accelerated growth observed in implanted pancreatic tumours.

Tumour associated macrophages are gaining increasing interest due to their role in modifying the immunosuppressive tumour microenvironment and supporting cancer cell survival. Macrophages display a high amount of plasticity and can adopt different phenotypes depending on the micro-environmental stimuli they are exposed to. In the context of cancer, studies have demonstrated phenotypically distinct macrophage subpopulations[357] and although it is likely that subpopulations have different functions, studies fully exploring these potential diverging roles are currently lacking. We show that analysis of disaggregated orthotopically implanted pancreatic tumours by flow cytometry identified four different sub-populations of macrophages; ($CD45^+CD11b^{hi}F480^+Ly6C^+$, $CD45^+CD11b^{hi}F480^+Ly6C$, $CD45^+CD11b^{lo}F480^+Ly6C^+$ and $CD45^+CD11b^{lo}F480^+Ly6C^-$), based on separation of populations seen on analysis. FACS separation and further *in-vitro* functional assays of these identified populations were not performed in this study, however further flow cytometry analysis revealed two main additional findings: FAK-depletion in pancreatic cancer cells results in M1-like polarisation of $Ly6C^+$ tumour associated macrophages, and reduced surface expression of the immune checkpoint ligand PD-L2 in all tumour associated macrophages.

Though there is some debate around the existence of alternatively activated/ M2 and classically activated/ M1 macrophage phenotypes[358], it is generally thought that the M2 phenotype is pro-tumour and the M1 phenotype is tumouricidal[61]. Tumours are often driven to a T_H2 polarised TME which results in escape from immune surveillance[350]. IL4 and IFNγ are two of the main drivers of the T_H2 and T_H1 immune responses respectively and likewise, are drivers of respective M2 and M1 macrophage polarisation[61]. In PDAC, 85% of all TAM are of the M2 phenotype[64] and their increased numbers correlate with increased tumour size and shorter survival times in post surgical patients[65]. Therefore the changes I observe in the

polarization of macrophages are likely to contribute to the tumour growth delay and increased survival in mice with FAK-/- tumours.

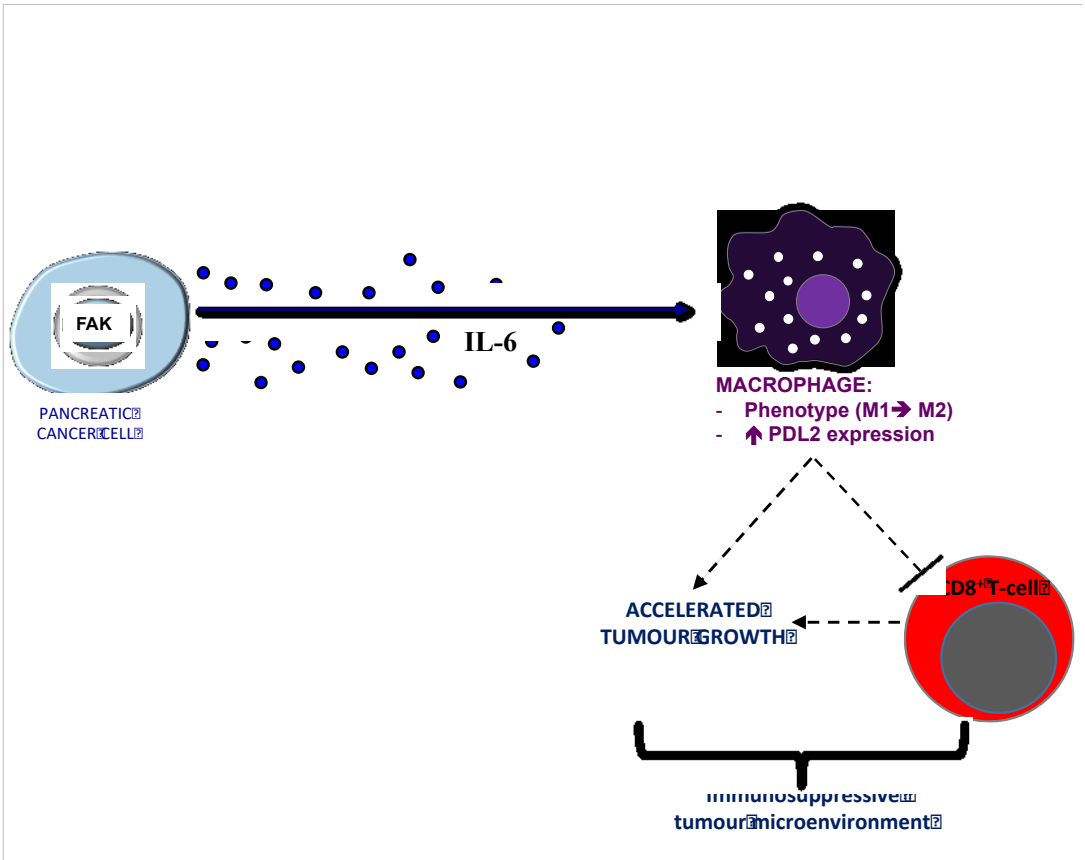


Figure 4.8 FAK signalling in pancreatic cancer cells regulates mechanisms of immune evasion to promote tumour growth (conclusions so far)

FAK-regulated cancer cell derived IL6 to reprogram tumour associated macrophages to a phenotype associated with tumour promotion, thus creating an immunosuppressive tumour microenvironment, with inhibition of cytotoxic T-cells and acceleration of tumour growth.

The PD-L1/PD-1 pathway has been extensively studied in the context of cancer and therapies inhibiting this pathway are currently undergoing clinical trials. Less emphasis has been placed on PD-L2, the other PD-1 ligand; the role of up-regulated PD-L2 in modulating tumour immunity is less well understood. Compared to PD-L1, constitutive basal expression of PD-L2 is low and expression is largely restricted to antigen presenting cells such as myeloid cells and DCs[350] but it has been reported on some other cell types. Both PD-L1 and PD-L2 are over expressed in human PDAC cancer[258]. Despite this, PD-1 inhibition has yet to yield a therapeutic advantage in clinical trials. A reduced tumour growth rate however was recorded when mice bearing Pan02 tumours were treated with PD-L2 blocking

antibodies, and this was comparable to that seen when PD-L1 or PD-1 was inhibited[359], suggesting that PD-L2 inhibition can effectively induce anti-tumour responses, even in the absence of PD-L1 inhibition. On the one hand this may reflect targeting of PD-L2 is a potential untapped therapeutic option, however the model used should also be taken into consideration. The Pan02 cell line is derived from PDAC tumours arising in the pancreas of C57BL/6 mice implanted with 3-methyl-cholanthrene (3-MCA)-saturated threads of cotton in the pancreas[360]. This carcinogen induced cell line model reproduces human PDAC histologically but it lacks some of the most common driving mutations present in human PDAC: *Kras* and *Trp53*[361], and it may also generally harbour a higher mutational burden as a consequence of the way in which it was derived. A higher neoantigen level could potentially make this model more immunogenic, therefore lacking clinical significance when compared to the low immunogenicity of the majority of human PDAC. Thus the jury is still out as to whether a clinical advantage can be gleaned from inhibiting the PD-L1/PD-L2-PD-1 axis in pancreatic cancer.

PD-1 may not be the only receptor by which PD-L2 exerts its effects in T-cell activation. Murine models of allergy show more severe disease when PD-L2 is inhibited but not PD-L1, and PD-L2 can still exert functional effects on T-cells derived from PD-1 deficient mice[362,363]. It may be that therapeutic approaches targeting PD-L2 remains an untapped avenue for treatment of pancreatic cancer.

In recent years several groups have shown that murine macrophage PD-L1 and PD-L2 expression is differentially regulated by T_H1 and T_H2 T cells respectively[348,350]. Various studies show that PD-L2 on macrophages is up-regulated in an immunosuppressive environment in response to T_H2 cytokines such IL10[364], and IL4[365]. A T_H2 polarised microenvironment contains the same environmental stimuli required for M2 polarisation of TAMs. It is therefore not surprising that both M2 macrophage polarisation and PD-L2 up-regulation were found concurrently on PDAC TAMs. FAK appears to be inducing PD-L2 across the tumour myeloid populations but only induces MMR expression in $Ly6C^+$ macrophages. This suggests that MMR and PD-L2 are regulated by independent mechanisms.

As shown in this chapter, cancer cell FAK signalling appears to enhance both M2-like macrophage polarisation and myeloid compartment PD-L2 expression. Therefore, our next line of enquiry was to investigate whether cancer cell FAK signalling is responsible for alteration in cancer cell derived cytokines expression, including production of T_H2 cytokines, and whether there was any subsequent change in M2 macrophage polarisation and PD-L2 expression.

5 FAK-dependent IL6 shifts the TAM phenotype to the pro-tumour phenotype and regulates pancreatic tumour growth *in-vivo*

5.1 Introduction

The phenotype of tumour associated macrophages (TAMs) can be shaped by signals they receive from their surrounding microenvironment[61]. Examples of these signals include cytokines such as, but not limited to, IL4, CSF-1, IL13, IL10[60,67], IL6[366,367], G-CSF[368,369] and GM-CSF[76,370,371]. FAK has previously been shown to regulate tumour-derived factors that can influence the composition of the TME within various cancer models including SCC[197] and pancreatic cancer[142]. In SCC, this immune cell TME regulation was found to be dependent upon both FAK kinase activity and also nuclear translocation of FAK, where FAK regulated transcription of various chemokines and cytokines. CCL5 expression in SCC cancer cells led to elevated levels of Tregs in the TME and subsequent suppression of the anti-tumour $CD8^+$ T-cell response, permitting growth of FAK-expressing tumours[197]. I therefore hypothesised that FAK-dependent regulation of paracrine signalling represents a potential mechanism through which FAK signalling in pancreatic cancer cells can influence the phenotype of TAMs.

5.1.1 Aims

1. To determine whether FAK regulates chemokine / cytokine secretion from pancreatic cancer cells, and if so:

2. Determine whether FAK-dependent paracrine signalling regulates the TAM phenotype and pancreatic tumour growth.

5.2 Results

5.2.1 FAK regulates chemokine / cytokine secretion from pancreatic cancer cells.

To investigate how FAK expression in Panc47 cells might regulate the phenotype of TAMs in pancreatic tumours, I first sought to determine whether FAK regulates the secretion of chemokines and cytokines from pancreatic cancer cells. Using forward-phase protein arrays (FPPA) that enabled the measurement of a set of 44 chemokines/cytokines present in cancer cell conditioned media (**table 5.1**), I identified that loss of FAK expression in Panc47 cells resulted in broad reprogramming of chemokine/cytokine secretion (**figure 5.1A**). Data for differentially expressed genes were median centred and subjected to unsupervised agglomerative hierarchical clustering on the basis of Euclidean distance, computed with a complete-linkage matrix, using Cluster 3.0 (Clustering Library, *version 1.37*) [372]. Clustering results were visualized using Java TreeView (*version 1.1.1*)[373].

A number of these FAK regulated cytokines including IL6[366,374], G-CSF[368,369], GM-CSF[370,371,375] have been shown previously to influence macrophage proliferation and function. I initially selected IL6 as a promising candidate for further investigation due to the well-established links between IL6 over-expression and pancreatic cancer progression. In the clinical setting, increased serum levels of IL6 or IL6 overexpression in patients with pancreatic cancer is associated with reduced survival, cancer cachexia and increasing tumour stage[93,171,376,377]. In the pre-clinical setting, several studies utilising KRAS-induced models of murine PDAC have found increased IL6 is required for PDAC progression[93,181,182,378]. To confirm the results from the FPPA, Panc47 FAK-wt and FAK-/- cells were cultured *in-vitro* for 48 hours, and an IL6 specific ELISA used to measure IL6 levels in cancer cell conditioned media (**figure 5.1B**). These results confirmed that FAK loss resulted in reduced IL6 secretion. Given that in the *in-vivo* environment, cancer cells will be subject to stimulation by other cytokines that might alter IL6 expression, I also investigated FAK-dependent IL6 regulation following stimulation with IL17, a potent inducer of IL6 expression[114,379–382] that is also present in the pancreatic tumour microenvironment[93,383]. Indeed IL17 secreted by Th17 cells[384] can promote initiation

and progression of PDAC[383,385] and the pancreatic stem cell niche[386]. Panc47 FAK-wt and FAK-/- cells were cultured *in-vitro* and stimulated with the recommended reported dose in the literature for stimulation of cultured cell lines *in-vitro* at 10ng/ml IL17[387], for 48hours. An IL6 specific ELISA was used to measure IL6 concentration in conditioned media (**figure 5.1C**). These results further confirmed that even in the presence of a strong stimulus, FAK loss results in a significant reduction in IL6 secretion. Furthermore, the extent of FAK-dependent IL6 regulation was not dependent on the concentration of IL17 used (**figure 5.2A**), neither was it due to differences in IL17 receptor surface expression on Panc47 FAK-wt and FAK-/- cells (**figure 5.2B**). Therefore, FAK regulates IL6 secretion under both normal culture conditions and following stimulation with IL17.

FPPA CAPTURE ANTIBODIES			
CCL11	CCL5	IL3	TREM 1
CCL12	CCL6	CCL22	COMPLEMENT
CCL2	CCL9	CCL27	CX3CL1
CCL21	CHEMERIN	CCL28	CXCL1
CXCL11	GM-CSF	CCL3	CXCL10
CXCL12	ICAM-1	CXCL9	IL12
CXCL13	IFNγ	CCL1	IL13
CXCL16	IL-1α	CCL17	IL17
IL1β	M-CSF	G-CSF	IL1RA
IL10	TIMP-1	IL4	IL6
IL2	RANK-L	IL5	IL7

Table 5.1 List of FPPA capture antibodies

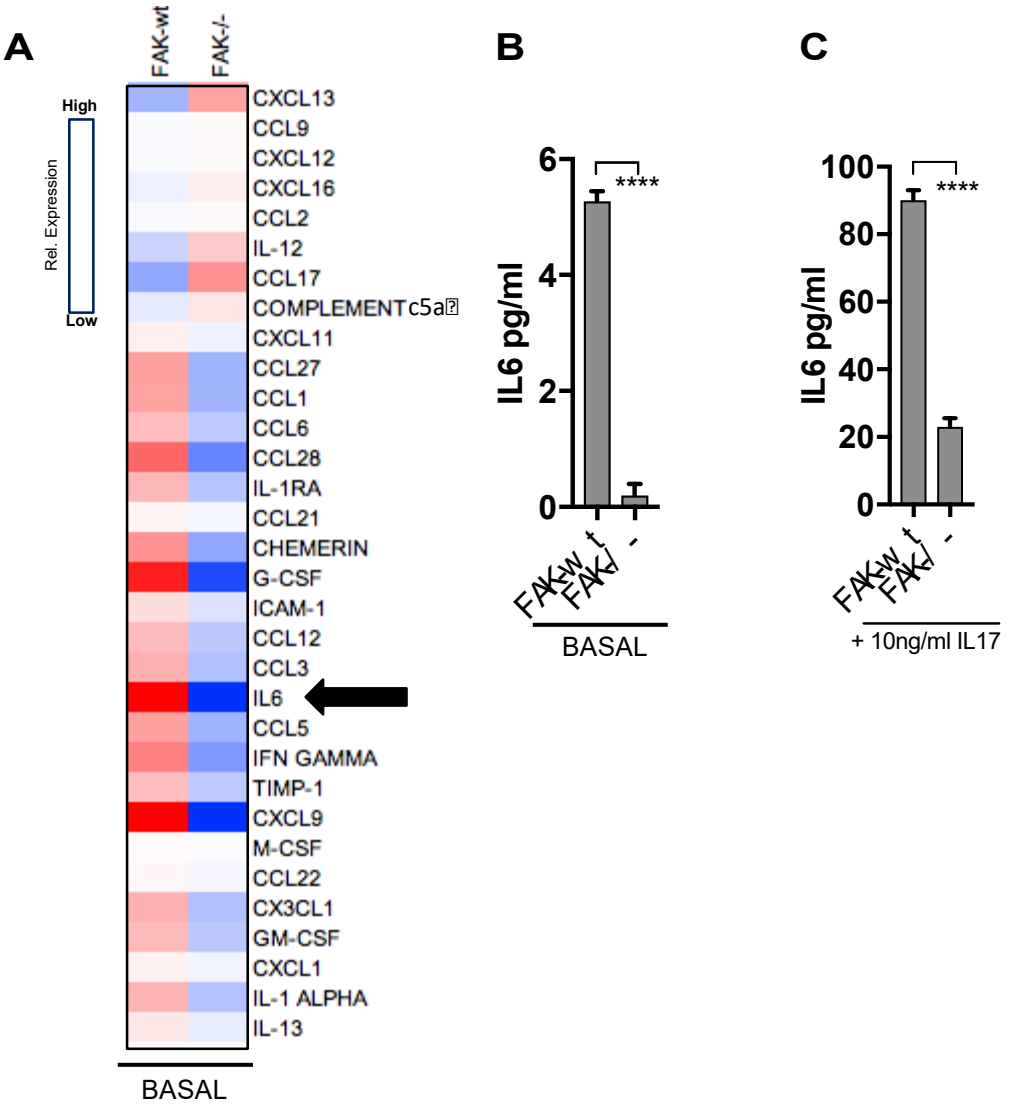


Figure 5.1 FAK regulates the secretion of a number of chemokines and cytokines including IL6 in Panc47 pancreatic tumour cell lines

A FFPA heat map generated by hierarchical clustering of changes between Panc47 FAK-wt and FAK-/- tumour cell lines. Colour and intensity indicate relative protein expression levels in culture medium. Over-expressed cytokines (red) include IL6, G-CSF, GM-CSF, CCL28 and CXCL-9 (data analysis for figure courtesy of Dr Alan Serrels) **B** Panc47 FAK-wt and FAK-/- cells were cultured *in-vitro* and basal IL6 production was measured from supernatants using ELISA **C** Panc47 FAK-wt and FAK-/- cells were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours, IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of three experiments; statistical analysis was performed using Student's T test; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

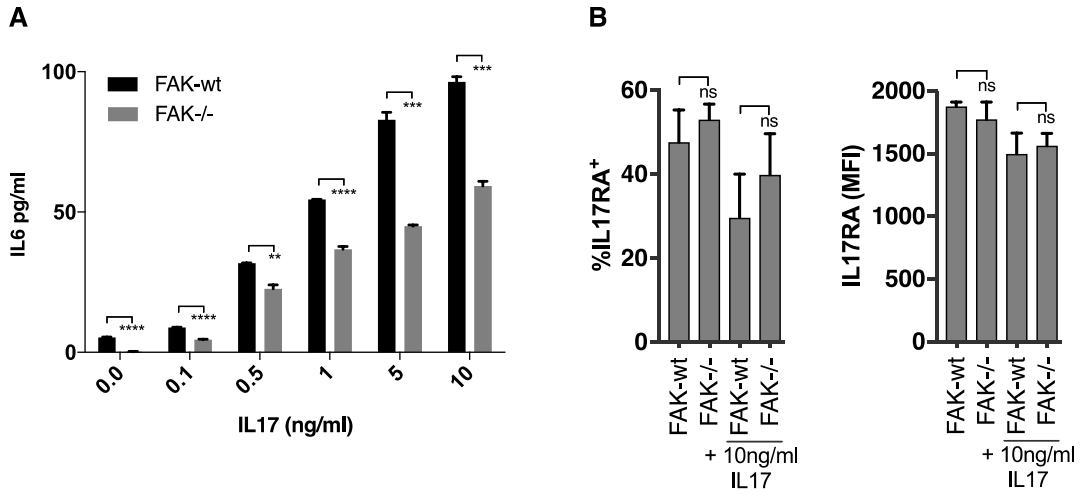


Figure 5.2 FAK regulates secretion of IL6 from Panc47 pancreatic tumour cells after stimulation at a range of IL17 concentrations. IL17 receptor expression is independent of FAK expression and IL17 stimulation

A Panc47 FAK-wt and FAK-/- cells were cultured *in-vitro* with increasing concentrations of IL17 for 48 hours; IL6 production was measured from supernatants using ELISA **B** Panc47 FAK-wt and FAK -/- pancreatic tumour cells were culture *in-vitro* with or without IL17 stimulation at 10ng/ml for 48 hours and surface IL17 receptor expression and MFI (mean fluorescence intensity) was analysed with flow cytometry. Error bars represent SEM, data are representative of three experiments; statistical analysis was performed using Student's T test and one-way ANOVA respectively; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

5.2.2 FAK-kinase activity is required for secretion of IL6

FAK is a protein tyrosine kinase and many of its cellular functions including cell survival, motility, invasion, epithelial-to-mesenchymal transition (EMT), angiogenesis and chemokine/ cytokine secretion[142,197,263,388] are regulated by its kinase activity. FAK also has kinase-independent scaffolding functions through FERM domain interactions by acting as a bridge between proteins or as a platform for assembly of protein complexes[389]. These can also contribute to FAK-dependent regulation of cell migration, cell polarity, survival, proliferation and cancer metastasis[267,277]. In order to determine whether IL6 regulation was dependent on FAK kinase activity, I expressed a kinase-deficient FAK mutant into Panc47 FAK-/- cells (KD G431). This allowed delineation between FAK kinase dependent and independent functions, enabling me to further explore the mechanism underpinning FAK-dependent IL6 expression.

FAK exists in an auto-inhibited state through intra-molecular FERM-kinase domain interactions. Disruption of these interactions leads to phosphorylation of tyrosine 397 (pY397) by the FAK kinase domain. Auto-phosphorylation of pY397 leads to activation of FAK's intrinsic kinase function and downstream kinase-dependent signalling pathways[268,390]. The use of antibodies specific to both total FAK and the auto- phosphorylation site pY397 demonstrated selective loss of FAK kinase in the KD G431 mutant cell line (**figure 5.3A**). Analysis of IL6 protein secretion using ELISA analysis revealed reduced IL6 secretion from KD G431 cells, comparable to that of FAK-/- cells (**figure 5.3B**). Thus FAK kinase activity is required for FAK-dependent regulation of IL6 secretion.

A number of small molecule FAK kinase inhibitors are currently being tested in early-phase (I/II) clinical trials. I therefore used one of these inhibitors, *BI853520*[306,307], to investigate whether pharmacological inhibition of FAK kinase activity could also reduce IL6 secretion from Panc47 FAK-wt cells. To determine the optimal the dose of *BI853520* required to selectively inhibit FAK kinase activity in Panc47 FAK-wt cells *in-vitro,* I treated cells with increasing doses of *BI853520* and used FAK auto-phosphorylation of tyrosine 397 as a readout of kinase inhibition using western blotting (**figure 5.4A**). Quantification of the fluorescent intensity of the band corresponding to FAK Y397 on the western blot identified 100nM as the

lowest concentration required to achieve maximal de-phosphorylation of FAK (**figure 5.4B**). In agreement with the IL6 ELISA analysis utilising the FAK kinase deficient cell line (KD G431), treatment of Panc47 FAK-wt cells with 100nM *BI853520* resulted in a reduction of IL6 secretion comparable to that of FAK-/- cells (**figure 5.5C**).

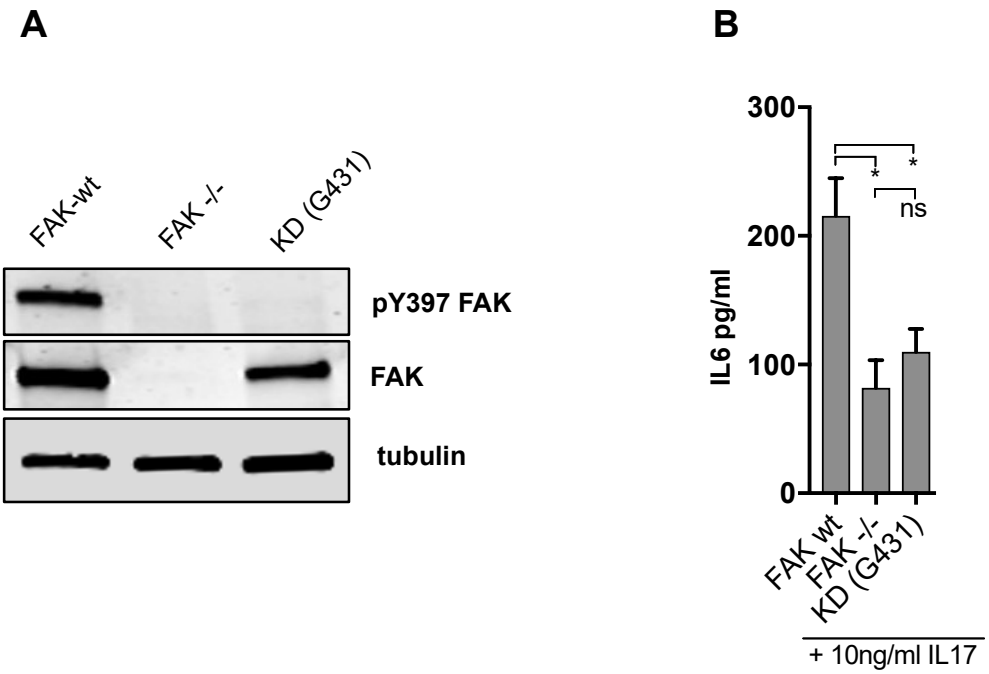


Figure 5.3 FAK's regulation of IL6 in Panc47 pancreatic tumour cells is a dependent on FAK's kinase function

A Western blot of total lysates from FAK-wt, FAK-/- and KD mutant (G431). Tubulin was used as a loading control. The western blot was probed with antibodies specific to FAK and pY397, demonstrating loss of pY397 in the KD mutant (KD G43). **B** Panc47 FAK-wt and FAK-/- and KD G431 pancreatic tumour cells were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours. IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of one experiment with two biological replicates; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

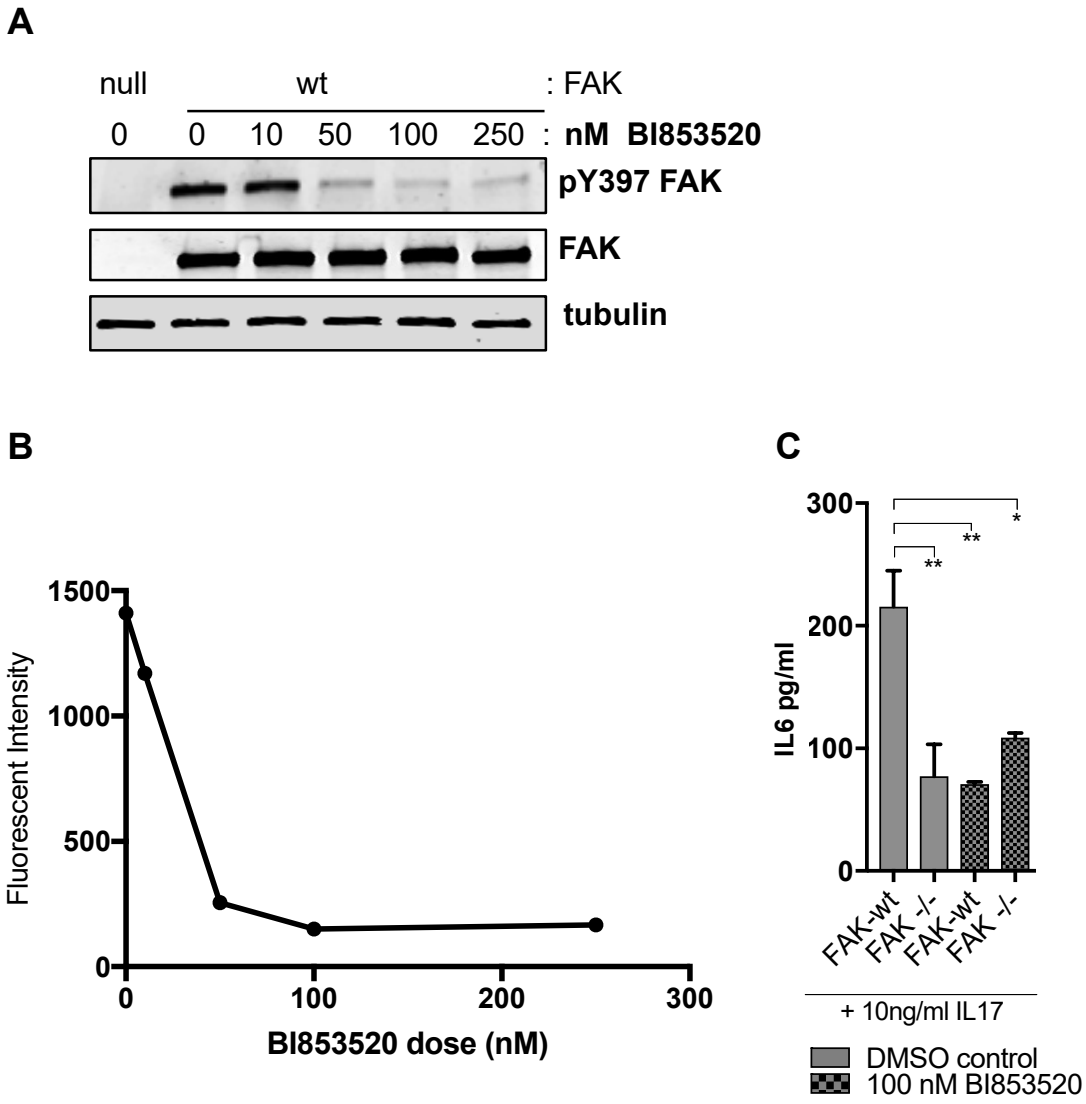


Figure 5.4 IL6 secretion from in Panc47 pancreatic tumour cells is regulated by FAK kinase inhibitor BI1853520 *in-vitro*

A Dose determination of FAK kinase inhibitor *BI853520.* Representative western blot of total lysates from FAK-wt cell lines. The FAK kinase inhibitor *BI853520* was added to supernatants for 48 hours at increasing doses ranging from 10nM to 5μM in order to establish the minimum dose required to dephosphorylate FAK. FAK-/- cells were used as a negative control and tubulin was used as a loading control. **B** Graph of fluorescent intensity from WB analysis against FAK kinase inhibitor *BI853520* dose, demonstrating maximal dephosphorylation begins at 100nM. **C** Panc47 FAK-wt and FAK-/- were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours, with or without the addition of 100nM *BI853520*. IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of one experiment with two biological replicates; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

5.2.3 Nuclear localisation of FAK is required for secretion of IL6

Having established that FAK kinase activity was required for FAK-dependent IL6 secretion, I next investigated whether FAK nuclear localisation was also important. The N-terminal FERM (band 4.1, ezrin, radixin, moesin homology) domain consists of 3 subdomains; F1, F2, and F3. The F2 lobe of the FERM domain contains nuclear localisation sequences required for FAK to shuttle from its cytoplasmic position at sites of focal adhesions to the nucleus[277] where it complexes with transcriptional regulators[290,292,293,391]. Nuclear FAK has been found to have a number of functions including transcriptional control of chemokines/ cytokines CCL5 and TGFβ in SCC to promote an immunosuppressive TME[301], suppression of GATA4 to repress VCAM-1 expression and enhancement of proteosomal degradation of TP53 to promote cell survival[291]. Additionally, nuclear FAK correlates with reduced survival in patients with colorectal cancer[392]. To investigate FAK nuclear function, I expressed a mutant FAK deficient in nuclear targeting[197,393] into Panc47 FAK-/- cells. Biochemical fractionation to separate nuclear and cytoplasmic extracts followed by western blotting of protein extracts confirmed that the FAK-NLS mutant cell line was defective in nuclear FAK (**figure 5.5A**). Subsequent ELISA analysis identified reduced IL6 secretion from Panc47 FAK-NLS cells, comparable to that secreted by the FAK-/- cells (**figure 5.5C**). Furthermore, implantation of 0.5 x 10^6 Panc47 FAK-NLS cells into the pancreas of C57BL/6 mice identified a similar reduction in growth to that observed with Panc47 FAK-/- cells (**figure 5.5B**). Thus, I can conclude from this and the results presented in 5.2.2 that FAK nuclear translocation promotes growth of Panc47 tumours and regulates IL6 secretion in a kinase-dependent manner.

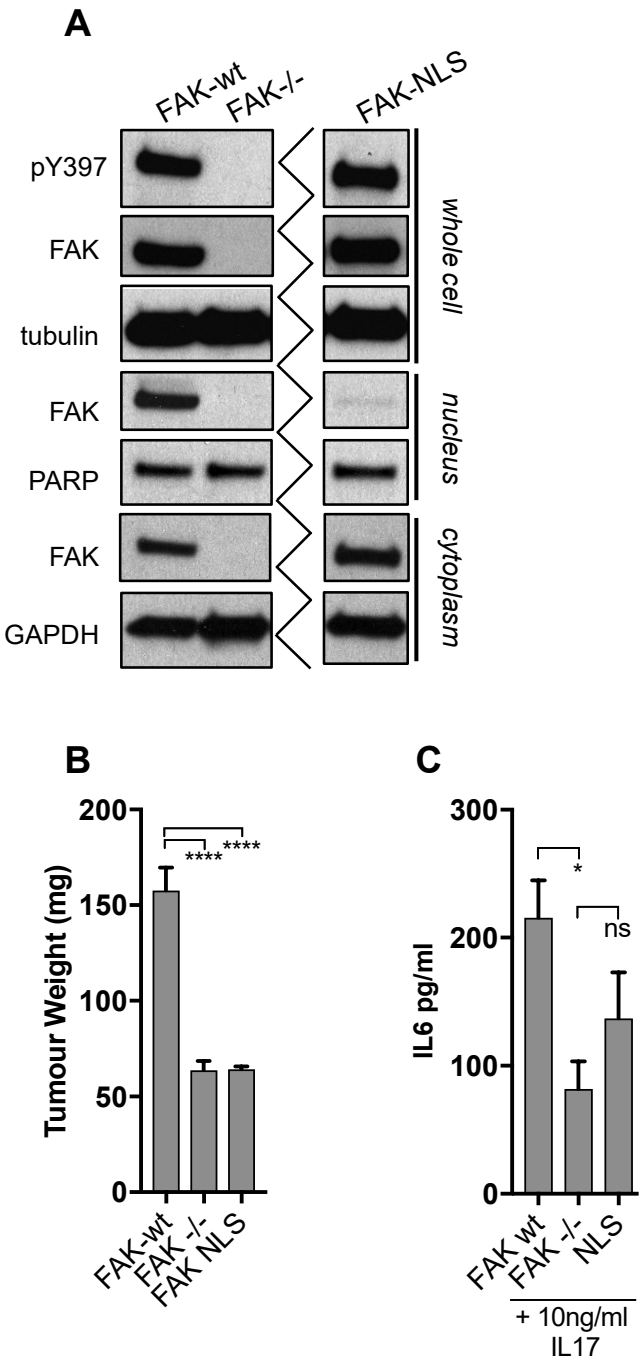


Figure 5.5 FAK regulates transcription of IL6 and this is partly dependent on FAK's nuclear translocation

A Western blot of total, nuclear and cytoplasmic fractions obtained after nuclear fractionation of FAK-wt and NLS mutant pancreatic cell line. FAK-/- acts as a negative control. Western blot demonstrates comparable levels of total FAK in FAK-wt and NLS mutant lines and absence of nuclear FAK in the NLS mutant line. The western blot was probed with antibodies specific to FAK, nuclear PARP and cytoplasmic GAPDH, demonstrating efficient fractionation and the presence of

cytoplasmic and nuclear FAK **B** The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt, FAK-/- or FAK NLS cell lines. Tumours were harvested at two weeks (n= 7 mice per group; error bars represent SEM; statistical test used was Students T-test) **C** Panc47 FAK-wt, FAK-/- and NLS pancreatic tumour cells were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours. IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of one experiment with two biological replicates; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001

5.2.4 FAK-regulated secretion of IL6 controls pancreatic tumour growth, the M2 tumour macrophage phenotype and PD-L2 expression *in-vivo*

Having established that FAK expression in Panc47 cells plays a role in regulating pancreatic tumour growth and the TAM phenotype, I next set out to determine whether FAK-regulated IL6 plays a role in any of these outcomes. IL6 mediates several important physiological functions: the acute phase inflammatory response, cell growth, survival and regulation of B-cells and T-cell differentiation and activation[394]. Most notably, IL6 is widely reported to tip the infiltrating leucocyte T_H1/T_H2 balance towards T_H2 cells[94,162,172,394–396], cells well recognised for their tumour- promoting capabilities. Specifically in pancreatic cancer, tumour cell derived IL6 has previously been shown to regulate tumour growth through influencing various immune cell types within the TME. For example, Bellone el al demonstrated that pancreatic cancer cell derived IL6 can act together with IL10 and TGF-β to reduce activation and proliferation of DCs, resulting in a diminished anti-tumour response[396]. Conversely, Gnerlich et al. observed that overexpression of IL6 in a TGF-β secreting murine model of pancreatic cancer, can shift the balance from a Treg to a Th17 predominant environment, resulting in delayed tumour growth and improved survival[114]. Due to the known influence of IL6 on the immune populations within the TME, I therefore hypothesized that FAK-dependent expression of IL6 might play an important role in regulating Panc47 tumour growth through influencing the TAM phenotype. To investigate this further, and to dissect the function of FAK-regulated IL6 as opposed to other tumour promoting FAK functions, I first generated Panc47 FAK-wt cells in which IL6 expression was selectively depleted. To do this, I used lentiviral-mediated delivery of DNA constructs encoding shRNA specifically targeting IL6, and identified successful IL6 depletion using an IL6 specific ELISA assay. This approach resulted in the generation of two IL6 depleted Panc47 FAK-wt cell lines: FAK-wt IL6 shRNA1 and FAK-wt IL6 shRNA2. IL6 protein expression was reduced to a level comparable to that of the FAK-/- cell line. A non-targeting control (NTCO) line was created to ensure that any effects observed were due specifically to IL6 depletion. Western blotting demonstrated comparable levels of FAK expression in all cell lines, confirming no alteration in FAK expression following IL6 depletion occurred (**figure**

5.6A). To complement this approach, I also generated an additional cell line in which IL6 was re-expressed into Panc47 FAK-/- cells, resulting in IL6 secretion at comparable levels to that of Panc47 FAK-wt cells. This was also achieved using lentiviral infection and validated with western blotting (**figure 5.9A**) and ELISA (**figure 5.9B**). To determine the contribution of IL6 to Panc47 tumour growth, $0.5x10^6$ FAK-wt, FAK-/-, FAK-wt NTCO, FAK-wt IL6 shRNA1 and FAK-wt IL6 shRNA2 cells were orthotopically implanted into the pancreas of C57BL/6 mice. Mice were culled 14 days later and their tumours removed and weighed. Comparison of tumour weights revealed that IL6 depletion in Panc47 FAK-wt cells resulted in a significant reduction in tumour weight, indicating IL6 plays an important role in promoting FAK-wt tumour growth (**figure 5.6C, figure 5.9C**). It should be noted that although the difference between the IL6 knockdown groups and the FAK-/- group was comparable and non-significant, the decrease in tumour size between the FAK-wt and FAK-/- is approximately 2.5 fold, whereas the decrease in tumour size between the FAK-wt and the IL6 knockdown lines is approximately 1.7 fold. Similarly, re-expression of IL6 into the FAK-/- pancreatic cancer cell line (FAK-/-IL6) restores growth of FAK-/- IL6 tumours to a level not significantly different to FAK-wt tumours, but does not fully restore the growth phenotype observed *in-vivo* (**figure 5.9C**). FAK has a plethora of functions in cancer and it is therefore not surprising that IL6 knockdown alone in FAK-wt tumours does not reduce the tumour growth to that of the FAK-/- tumours. I therefore conclude that pancreatic FAK-wt tumour growth *in-vivo* is partly dependant on cancer cell IL6 expression.

I next set out to determine whether FAK-IL6-dependent tumour growth was associated with regulation of the TAM phenotype. C57BL/6 mice were orthotopically implanted with $0.5x10^6$ FAK-wt, FAK-/-, FAK-wt NTCO, FAK-wt IL6 shRNA1 and FAK-wt IL6 shRNA2 cells, and tumours harvested at day 14, disaggregated and stained for flow cytometry analysis using an optimised panel of antibodies (**stain1**, **table 4.1**). Comparison between FAK-wt and FAKwt-NTCO with FAK-/- and the IL6 knockdown lines demonstrates a reduction in MMR^+ $CD86^-$ cells, implying a switch from M2-like to M1-like phenotype in the $Ly6C^+$ TAM population (**figure 5.7**) and a significant reduction in surface expression of PD-L2 in all TAM populations (**figure 5.8**). Re-expression of IL6 into the FAK-/- pancreatic

cancer cell line increased the proportion of MMR^{+} $CD86^{-}$ $Ly6C^{+}$ TAMs indicating a switch from the M1-like to M2-like phenotype (**figure 5.10A, 5.10B**). Hence, IL6 plays an important role in regulating the TAM phenotype, driving the expression of markers associated with a pro-tumour function and increased immuno-suppressive activity.

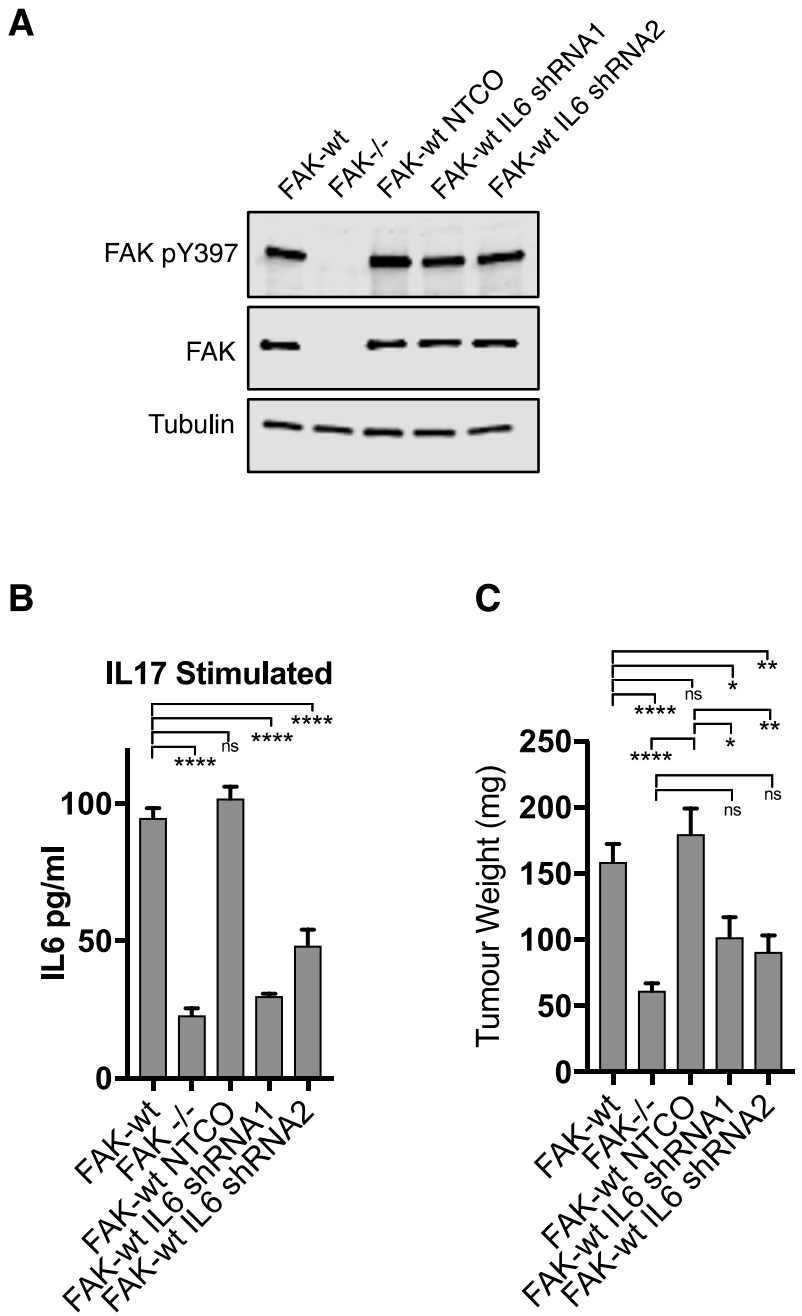


Figure 5.6 IL6 knockdown in Panc47 FAK-wt pancreatic tumour cells partly controls pancreatic tumour growth *in-vivo*

A Validation of FAK-wt IL6 shRNA lines and shRNA empty vector control (FAK-wt NTCO. Representative western blot of total lysates from Panc47 FAK-wt, FAK-/-, FAK-wt NTCO, FAK-wt IL6 shRNA1 and FAK-wt IL6 shRNA2. Tubulin was used as a loading control. The western blot was probed with antibodies specific to FAK and pY397, demonstrating comparable levels of FAK expression in all cell lines. **B** Panc47 FAK-wt, FAK-wt NTCO, FAK-/-, FAK-wt IL6 shRNA1 and FAK-wt IL6 shRNA2 cell lines were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours, IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of a combination of 5 experiments; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001. **C** The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ FAK-wt, FAK-wt NTCO, FAK-/- , FAK-wt

IL6 shRNA1 and FAK-wt IL6 shRNA2 cell lines derived from the LSL-Kras$^{G12D/+}$;LSL-Trp53$^{R172H/+}$ pancreatic cancer cell line. Tumours were harvested at two weeks (data are representative of combined experiments total n= 45 mice; error bars represent SEM; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001)

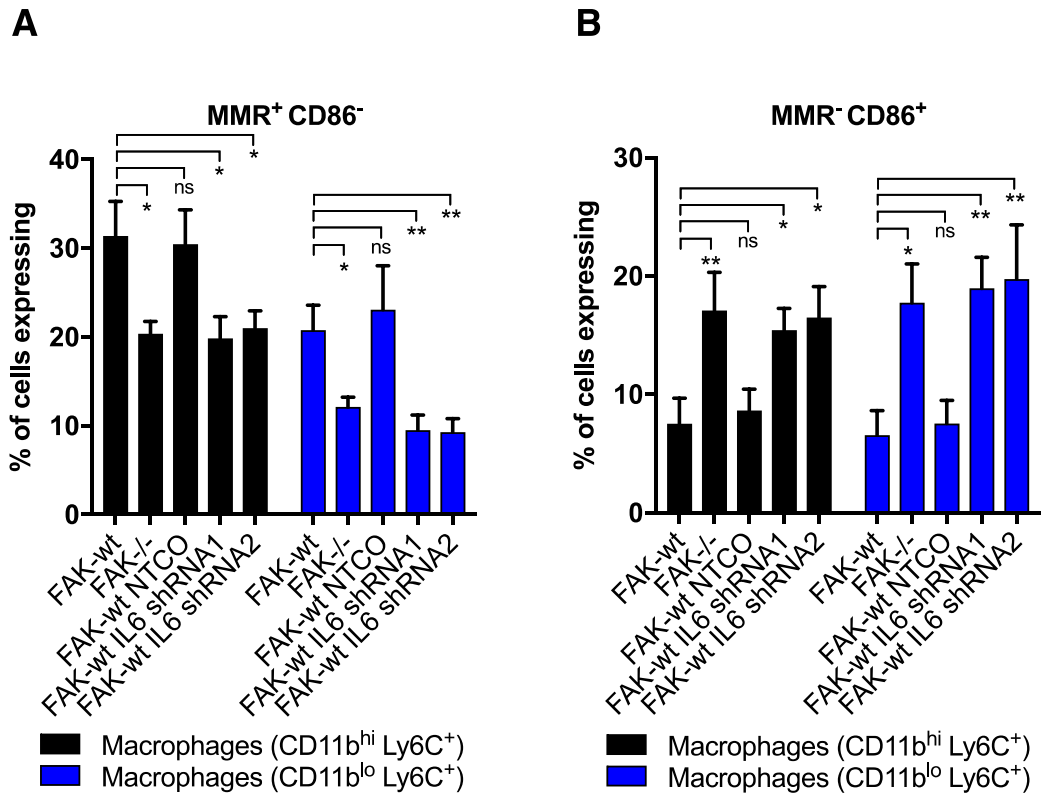


Figure 5.7 FAK regulated secretion of IL6 from Panc47 pancreatic tumour cells controls the Ly6C$^+$ tumour macrophage phenotype

The pancreas of C57BL/6 mice were implanted with 0.5x10^6 Panc47 FAK-wt, FAK-/-, FAK-wt NTCO, FAK-wt IL6 shRNA1, FAK-wt shRNA2 or FAK-/- cell lines. Tumours were harvested at 14 days post implantation (data represents combined experiments n= 6 per group; error bars represent SEM; statistical test used was one-way ANOVA, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.2) **A** M2 (CD86$^+$ MMR$^-$) macrophages in CD11b^{hi}Ly6C$^+$ and CD11b^{lo}Ly6C$^+$ TAMS. B M1 (CD86$^+$ MMR$^-$)) macrophages in CD11b^{hi}Ly6C$^+$ and CD11b^{lo}Ly6C$^+$ TAMSCD11b^{hi}Ly6C$^+$ TAMs **B** M2 (CD86$^+$ MMR$^-$) and M1 (CD86$^+$ MMR$^-$) macrophages in CD11b^{hi}Ly6C$^+$ and CD11b^{lo}Ly6C$^+$ TAMSCD11b^{hi}Ly6C$^+$ TAMs.

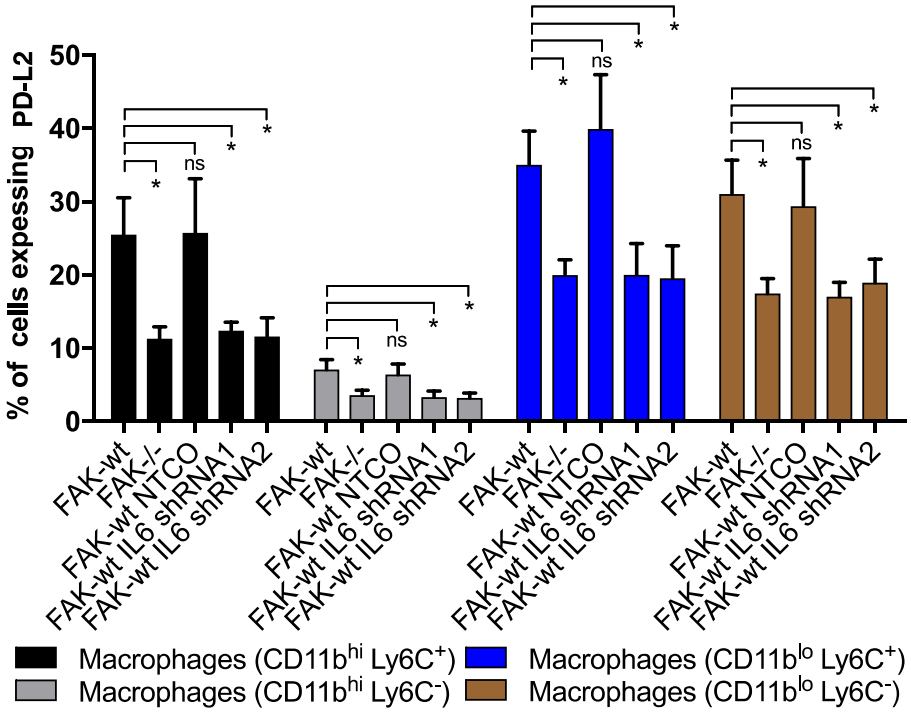


Figure 5.8 FAK regulated secretion of IL6 from Panc47 pancreatic tumour cells controls PD-L2 expression in all tumour associated macrophage subsets

The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt, FAK-/-, FAK-wt NTCO, FAK-wt IL6 shRNA1, and FAK-wt shRNA2 cell lines. Tumours were harvested at 14 days post implantation. (Data represents combined experiments n= 6 per group; error bars represent SEM; statistical test used was one-way ANOVA, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.3) Graph shows % PD-L2 expression on all four TAM subsets; $CD45^+CD11b^{hi}F480^+Ly6C^+$ TAM, $CD45^+CD11b^{hi}F480^+Ly6C^-$, $CD45^+CD11b^{lo}F480^+Ly6C^+$, $CD45^+CD11b^{lo}F480^+Ly6C^-$ TAM

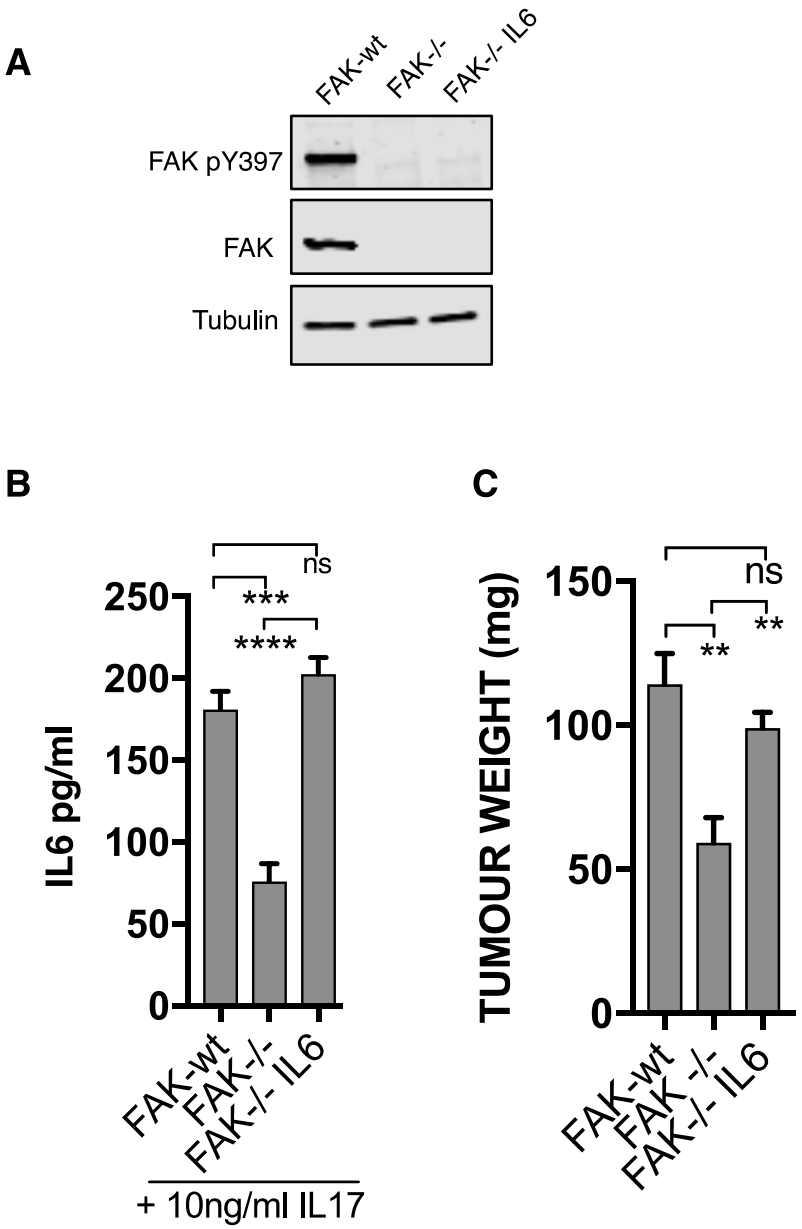


Figure 5.9 Re-expression of IL6 into FAK-/- Panc47 pancreatic tumour cells partially restores tumour growth *in-vivo*

A Validation of Panc47 FAK-/- IL6 lenti re-expression line. Representative western blot of total lysates from Panc47 FAK-wt, FAK-/- and FAK-/- IL6 lenti re-expression line. Tubulin was used as a loading control. The western blot was probed with antibodies specific to FAK and pY397, demonstrating comparable levels of FAK expression in all cell lines. **B** FAK-wt, FAK-/- and FAK-/- IL6 cell lines were cultured *in-vitro* and stimulated with 10ng/ml IL17 for 48hours, IL6 production was measured from supernatants using ELISA. Error bars represent SEM, data are representative of a combination of 3 experiments; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001. **C** The pancreas of C57BL/6 mice were implanted with $0.5x10^6$ Panc47 FAK-wt, FAK-/- and FAK-/- IL6 lenti re-expression cell lines. Tumours were harvested at two weeks (n= 12 mice in total, 6 mice in the FAK-/- IL6 group and 3 in each of the FAK-wt and FAK-/- groups; error bars represent SEM; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001)

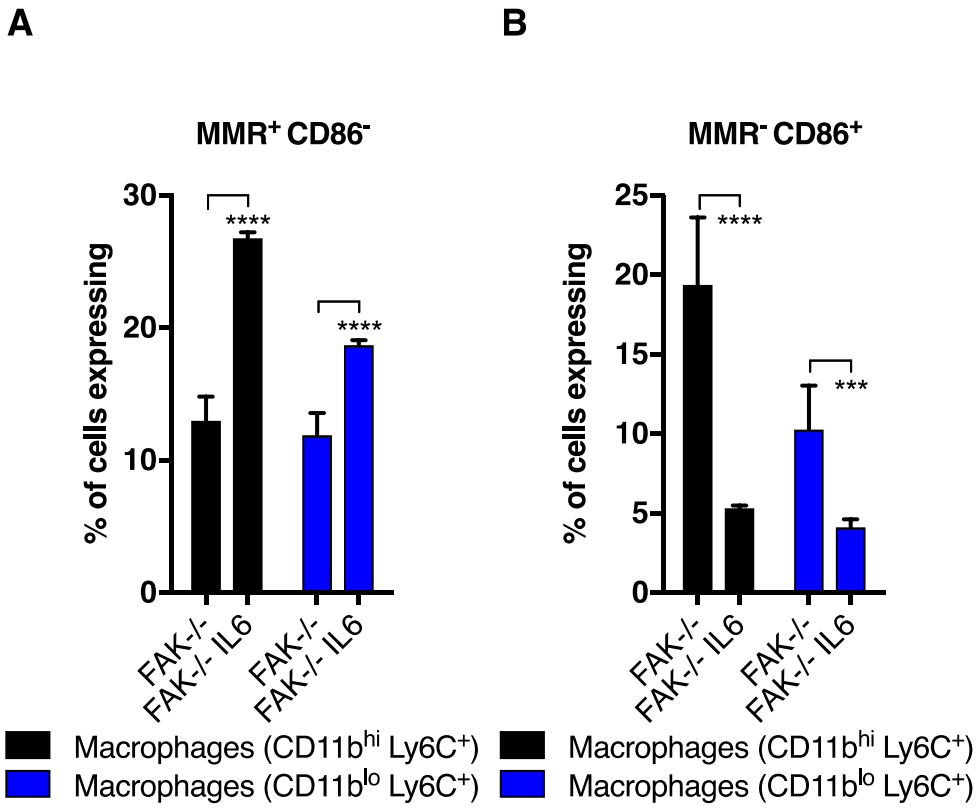


Figure 5.10 Re-expression of IL6 into FAK-/- Panc47 pancreatic tumour cells promotes re-polarisation of Ly6C$^+$ macrophages *in-vivo*

The pancreas of C57BL6 mice were implanted with 0.5x10^6 Panc47 FAK-/- and FAK-/- IL6 lenti re-expression cells. Tumours were harvested at two weeks (n= 9 mice in total, 6 mice in the FAK-/- IL6 group and 3 in the FAK-/- groups; error bars represent SEM; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001) **A** M2 (CD86$^+$ MMR$^-$) in CD11b$^{hi/lo}$Ly6C$^+$ macrophages in FAK-/- and FAK-/- IL6 lenti re-expression cell line tumours **B** M1 (CD86$^-$ MMR$^+$) CD11b$^{hi/lo}$Ly6C$^+$ macrophages in FAK-/- and FAK-/- IL6 lenti re-expression cell line tumours

5.3 Discussion

The central objective of this chapter was to determine whether FAK regulates chemokine / cytokine secretion from pancreatic cancer cells and whether any of these FAK-regulated tumour derived signals were responsible for controlling the pro-tumour associated TAM phenotype and the acceleration of pancreatic tumour growth observed *in-vivo*. A number of FAK-regulated cytokines were identified *in-vitro* using FFPA. IL6 was chosen as a target cytokine to further investigate due to its well described association with pancreatic tumour progression in both the clinical and pre-clinical setting[93,171,181,182,376–378]. I show that regulation of IL6 is dependent on FAK kinase activity and FAK nuclear localisation. Further I show that IL6 plays an important role in accelerating pancreatic tumour growth and regulating the TAM phenotype, driving the expression of markers associated with a pro-tumour function and increased immuno-suppressive activity *in-vivo.* Tumour size has not been controlled for in this series of experiments. Future experiments would include comparison between FAK-/- tumours and mice implanted with FAK-wt cell lines and treated with a FAK inhibitor drug to control for tumour size.

FPPAs are microarrays consisting of a defined set of immobilised capture antibodies, in this case targeting various inflammatory cytokines and chemokines, detected with a fluorescent dye-conjugated detection antibody to allow multiple targets to be analysed simultaneously. This method achieves a high degree of specificity[397] and an increased throughput speed at a reduced cost. It therefore represented a good method for screening several biomarkers at one time. Analysis of the resultant data identified over-expression of a number of cytokines/chemokines, most notably IL6, G-CSF, CM-CSF, CCL28 and CXCL-9 (**figure 5.1A**) at basal level *in-vitro.* FAK-dependent IL6 regulation was confirmed with ELISA analysis, and although expression of IL6 was detectable, it was at a low level. In the *in-vivo* setting, pancreatic tumour cells exposed to a variety of other cytokines that influence their behaviour, one of which is IL17[383–385]. Pancreatic cancer cells exposed to IL17 are induced to secrete IL6[114,379–382], a pathway principally regulated by NF-κB[384]. Given the low basal levels of IL6 secretion in the *in-vitro* environment, I opted to add IL17 to culture media to increase IL6 secretion and therefore increase sensitivity for analysis going forward. Despite IL17 being a robust IL6 stimulus, FAK depletion results in a significant reduction in

IL6 secretion. This regulation was not found to be dependent on levels of IL17 receptor expression on cancer cells but was found to be dependent on both nuclear shuttling and the kinase function of FAK.

Different drugs could preferentially target either the FAK-kinase or the kinase-independent scaffolding function[265]. Therefore an understanding of the mechanism behind FAK-dependent outcomes is important in both selection and development of appropriate drugs for pancreatic cancer treatment and interpretation of drug trials going forward. I show that expression of a catalytically inactive mutant FAK protein (KD G431) and treatment of FAK-wt cells with a small-molecule kinase inhibitor (*BI853520),* significantly inhibits IL6 production to levels comparable to that of Panc47 FAK-/- cells *in-vitro*. Further pre-clinical studies, including treatment of mice with small-molecule kinase inhibitors, should be employed to assess whether global FAK kinase inhibition results in the same outcome as specific inactivation of FAK kinase function within tumour cells only.

The *in-vitro* regulation of IL6 production was also found to be dependent on nuclear localisation of FAK in pancreatic cancer cells. In normal cells, nuclear FAK is absent or present at low levels and FAK has been shown to accumulate in the nucleus in response to cellular stress such as hypoxia[277], a condition common in the PDAC TME. Further, nuclear FAK has been identified as an indicator of poor prognostic outcome in colorectal cancer[392]. If nuclear FAK is specific to cancer cells, this may provide a new therapeutic opportunity; however further work will be required to establish the absence or level of nuclear FAK in normal pancreatic tissue.

I investigated the impact of IL6 on the *in-vivo* TAM populations using shRNA IL6 knockdown. Several approaches are available to induce targeted gene silencing or gene knockout in mammalian cells. shRNA knockdown was selected as the method of IL6 depletion. Using this method, target sequences are cloned into a lentiviral backbone. Once transfected into target cells, the viral vector containing the DNA target sequence, consisting of a sense and antisense copy of the target sequence separated by a loop sequence, is transcribed, exported from the nucleus, and the loop removed by a dicer. This results in two single stranded small interfering RNA (siRNA). The siRNA binds to the target mRNA with an identical sequence and is

degraded[398]. This was chosen as the preferred method rather than other gene modification methods such as CRISPR/Cas9 genome editing used previously, which achieves full gene knockout. Whilst infection with viral vectors allows for stable integration of shRNA, it does not completely shut off the gene; some functional RNA remains and is translated at lower levels, thereby reducing gene function but doesn't eliminate the gene altogether. The advantage of this is that IL6 production could be brought down to levels comparable to the Panc47 FAK-/- cell line, thus creating a more suitable and comparable model for investigation of FAK-dependent IL6 signalling in pancreatic tumours.

There are many cellular sources of IL6 within the PDAC TME: macrophages[93], epithelial cells, fibroblasts[182] and pancreatic cancer cells[114,170,181,183]. Here we show that IL6, specifically derived from pancreatic tumour cells, is crucial to shaping the immunosuppressive macrophage phenotype and subsequent control of tumour growth *in-vivo.* IL6 is already known to be fundamental to PDAC development and progression. It may be that tumour cells, although not the only producer of IL6, may instigate the cascade of phenotypic changes in the PDAC TAMs, resulting in an altered PDAC immune-TME. The identification of FAK-dependent cancer cell sourced IL6 in repolarising TAM to the pro-tumour phenotype enables a better understanding of the PDAC TME and may allow more effective immunotherapy strategies and/or aid personalised medicine strategies.

6 Cancer cell derived IL6 acts indirectly to modulate TAMs

6.1 Introduction

I have shown that FAK regulates pancreatic tumour growth and the TAM phenotype through a mechanism that is dependent on the action of IL6. IL6 has been associated with promoting the differentiation of TAMs to the pro-tumour M2 phenotype in pre-clinical murine lung cancer models[176]. However, T_H2 type cytokines such as IL4, IL10 and/or IL13 are far more widely reported to induce both M2 macrophages and PD-L2 expression[348,358]. Therefore, I set out to determine whether IL6 was acting in a direct manner from FAK expressing pancreatic cancer cells to macrophages, or whether there were intermediaries involved within the PDAC TME.

6.1.1 Aims

1. To establish whether FAK-wt pancreatic tumour cells directly induce the pro-tumour M2 macrophage phenotype *in-vitro* and if not:
2. To identify additional factors or cell types that might form part of this mechanism

6.2 Results

6.2.1 IL6 alone is not sufficient to drive expression of MMR and PD-L2 on TAMs

Having established that IL6 plays an important role in regulating the TAM phenotype *in-vivo*, I next wanted to determine whether this was a direct signalling mechanism between cancer cells and macrophages. To address this, Panc47 FAK-wt cells were cultured in normal growth media for 48 hours, and this media was transferred onto a culture of bone-marrow derived macrophages for a further 72 hours. Flow cytometry identified that Panc47 FAK-wt conditioned media (CM) was sufficient to induce expression of MMR but not PD-L2 in macrophages (**table 6.1, stain 3 and figure 6.1**). IL4 has been shown to polarise macrophages to the M2 phenotype and increase PD-L2 expression[349,399]. Analysis of FAK-wt and FAK-/-

CM with FFPA revealed that IL4 was either not expressed or not secreted at detectable levels. I therefore added IL4 to Panc47 FAK-wt conditioned media and measured MMR and PDL2 expression on BMDMs. These results identified that while CM alone can induce expression of MMR, it is enhanced by the addition of IL4. Furthermore, expression of PD-L2 requires IL4, but can be further enhanced by the addition of FAK-wt CM (**figure 6.1)**. Therefore, IL4 works in concert with secreted factors present in Panc47 FAK-wt CM to recapitulate the phenotype of macrophages observed in Panc47 FAK-wt tumours. To further probe the identity of the secreted factors enhancing the action of IL4 within FAK-wt conditioned media, I treated BMDMs with either IL4 + IL6, IL4 + G-CSF, or IL4 + GM-CSF, and determined the effects on MMR and PD-L2 expression using flow cytometry. IL6, G-CSF, and GM-CSF are all secreted by Panc47 FAK-wt cells and have been previously shown to regulate macrophage function[366,368–370,374]. These results identified that IL6 in combination with IL4 as the most potent combination in terms of MMR and PD-L2 up-regulation in BMDM (**figure 6.2**). These results imply that IL6 is not sufficient on its own to drive the changes in observed phenotype of macrophages in Panc47 FAK-wt tumours, and that it requires the action of IL4. This does not exclude the possibility that other cytokines may also be able to work in concert with IL6 to regulate the macrophage phenotype. I therefore sought to identify whether an accessory cell type, capable of IL4 production, was additionally required for FAK-dependent regulation of the macrophage phenotype.

STAIN 3	
MARKER	**FLUOROPHORE**
CD11b	BV421
F4/80	PR Cy7
MMR	PE
PD-L2	APC
Viability	Zombie NIR

Table 6.1 Flow cytometry marker panel used to identify BMDM MMR and PD-L2 expression

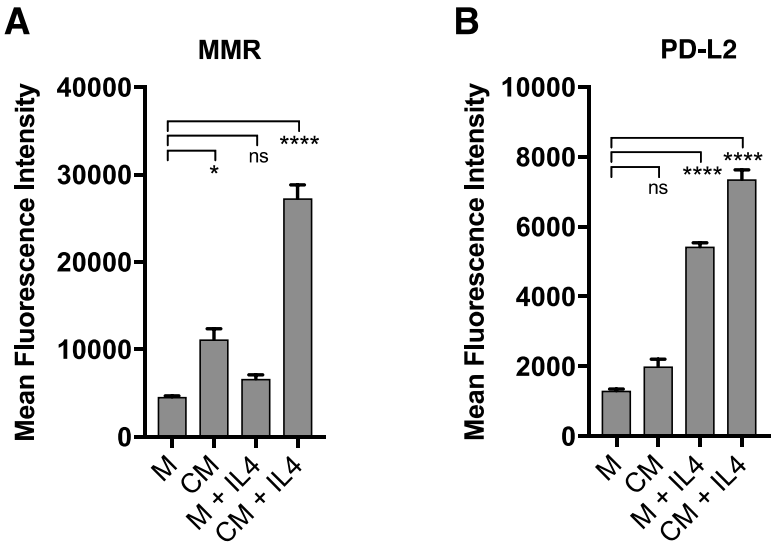


Figure 6.1 Panc47 FAK-wt CM requires the addition of IL4 to achieve high levels of MMR and PD-L2 expression on BMDMs

BMDM macrophages were cultured for 7 days in media (DMEM, M-CSF and10% FCS). Media was replaced with fresh DMEM + FCS (M), Panc47 FAK-wt media (CM) or Panc47 FAK-wt media with recombinant mouse IL4 (CM + IL4) for a further 24 hours. **A** MMR and **B** PD-L2 were measured with flow cytometry and are expressed as mean fluorescence intensity (MFI). Results represent n=3, error bars represent SEM; statistical analysis was performed using one-way ANOVA; P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001).

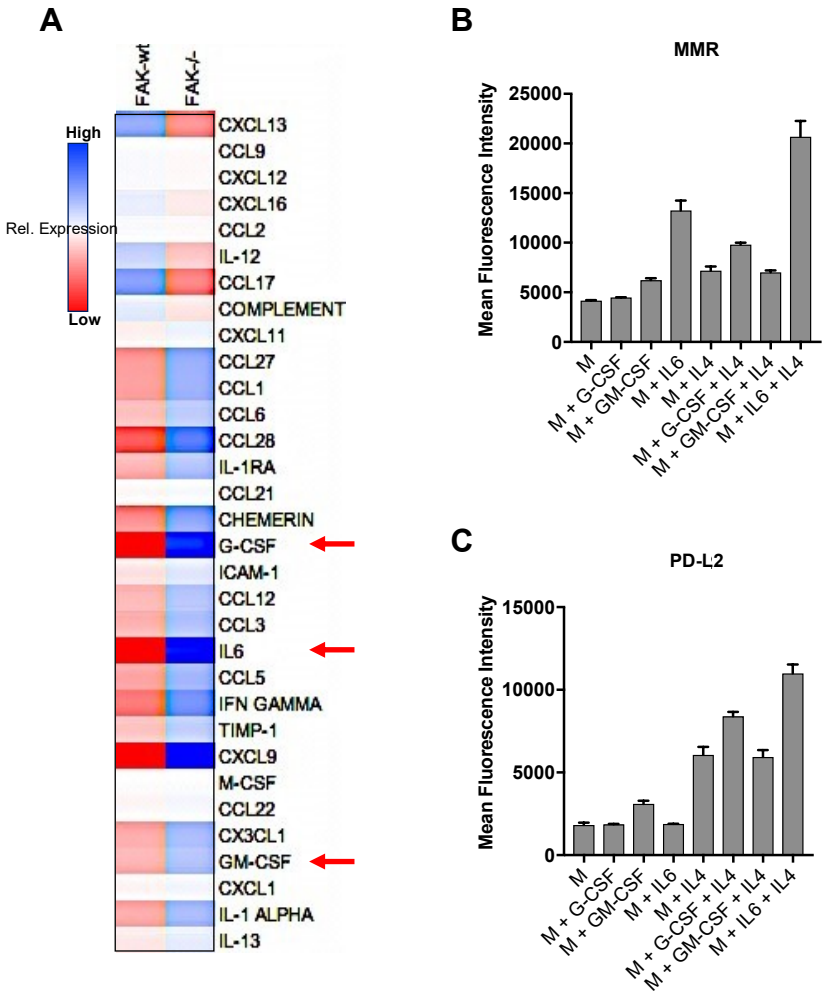


Figure 6.2 Effects of Panc47 FAK-wt expressed cytokines on MMR and PD-L2 expression in BMDMs

A Forward Phase Protein Array (FFPA) heat map generated by hierarchical clustering of changes between FAK-wt and FAK-/- tumour cell lines. Colour and intensity indicate relative protein expression levels in culture medium. Arrows indicate over-expressed cytokines (red) that are regulated by FAK and have been shown to play a role in macrophage polarisation (IL6, G-CSF, GM-CSF). BMDM macrophages were cultured for 7 days in media (DMEM, M-CSF and 10% FCS). Media was replaced with fresh DMEM + FCS (M), or M with recombinant mouse G-CSF, GM-CSF or IL6 at 20ng/ml for a further 24 hours. **A** MMR and **B** PD-L2 were measured with flow cytometry and are expressed as mean fluorescence intensity (MFI). Results represent n=3, error bars represent SEM

6.2.2 $CD4^+$ T-cells are necessary for FAK-mediated pancreatic tumour growth and the pro-tumour macrophage phenotype *in-vivo*

A candidate source of IL4 is T_H2 polarised $CD4^+$ T-cells. T_H2 polarised tumour microenvironments[358] and specifically IL4 produced from $CD4^+$ T-cells [64,400,401] have been shown to induce M2 TAM polarisation. Therefore to address whether $CD4^+$ T-cells are important for FAK-dependent pancreatic tumour growth acceleration and the pro-tumour macrophage phenotype observed in my model, I selectively depleted $CD4^+$ T-cells using an anti-$CD4^+$ T-cell depleting antibody in C57BL/6 mice, implanted with either Panc47 FAK-wt or FAK-/- cell lines. Tumour growth was significantly reduced, near that of the Panc47 FAK-/- tumours, in mice implanted with Panc47 FAK-wt tumours and treated with $CD4^+$ T-cell depleting antibody (**figure 6.2A**). No significant difference in tumour weight was observed between FAK-/- tumours from mice treated with either $CD4^+$ T-cell depleting antibody or isotype control. These results suggest that $CD4^+$ T-cells are important for FAK-dependent tumour growth. Further, I analysed the macrophage populations within these tumours (**stain 1, figure 6.2 B**). In Panc47 FAK-wt tumours harvested from mice treated with $CD4^+$ T-cell depleting antibody, there was a significant reduction in the percentage of macrophages expressing MMR and PD-L2, indicating a reduced number of M2-like/ pro-tumour associated macrophages within the TME when $CD4^+$ T-cells are absent. MMR and PD-L2 expression on macrophages from Panc47 FAK-/- tumours from mice treated with $CD4^+$ T-cell depleting antibody are comparable to those receiving isotype control. Therefore I conclude that $CD4^+$ T-cells play an important role in promoting Panc47 FAK-wt tumours growth, and are required to promote the pro-tumour associated macrophage phenotype observed in this model.

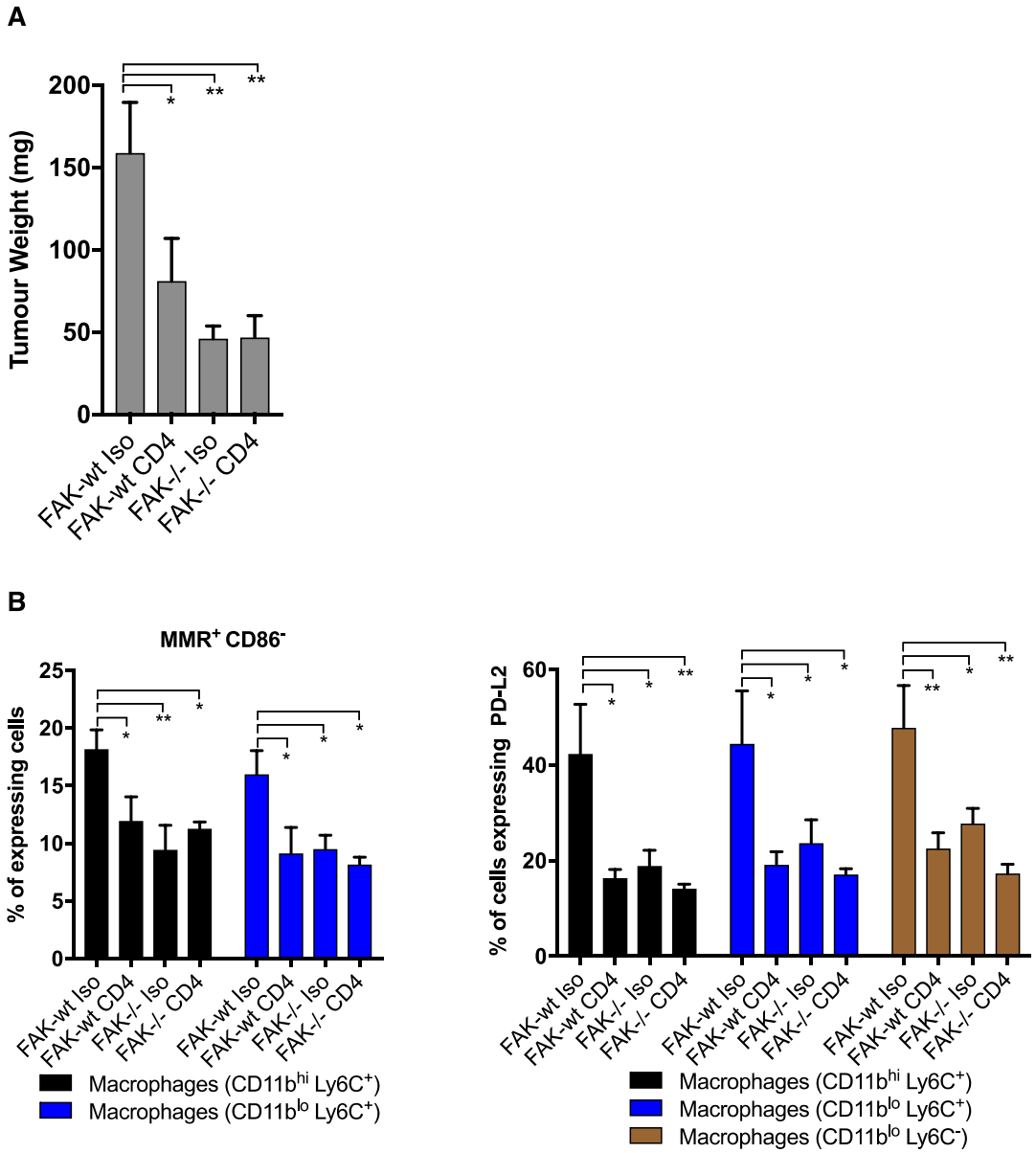


Figure 6.2 CD4$^+$ T-cells are required for FAK-dependent pancreatic tumour growth and reprogramming of the macrophage phenotype

The pancreas of C57BL/6 mice were implanted with 0.5x10^6 Panc47 FAK-wt or FAK-/- cells and harvested at 14 days post implantation. Mice were treated with either CD4 depleting antibody or isotype control (n= 12; 3 mice per group; error bars represent SEM; statistical test used was Students T-test, P-value = not significant >0.05, *<0.05, ** <0.01, ***<0.001, **** < 0.0001, gating as described in figure 4.2) **A** tumour weight at 14 days. **B** M2 (CD86$^-$ MMR$^+$) in CD11b$^{hi/lo}$Ly6C$^+$macrophages from Panc47 FAK-wt and FAK-/- pancreatic tumours in mice treated with CD4 depleting antibody or isotype control **C** PD-L2 expression in CD11b$^{hi/lo}$Ly6C$^+$ and CD11b$^{hi/lo}$Ly6C$^-$ macrophages from Panc47 FAK-wt and FAK-/- pancreatic tumours in mice treated with CD4 depleting antibody or isotype control

6.2.3 The secretome of Panc47 FAK-wt cells is sufficient to drive differentiation of naïve $CD4^+$ T-cells to the T_H2 phenotype

IL6 is a known to induce naïve $CD4^+$ T-cell to the T_H2 phenotype. This is through two independent mechanisms. Firstly by promoting early IL4 expression through up-regulation of Nuclear Factor of Activated T cells (NFAT)c2 expression. Secondly by rendering $CD4^+$ T-cells unresponsive to IFNγ signals through up-regulation of Silencer of Cytokine Signalling (SOCS)1 which leads to interference in IFNγ expression[94], the effector T_H1 cytokine. I have demonstrated a role for $CD4^+$ T-cells in pancreatic tumour growth and the requirement of both IL4 and IL6 in the polarisation of TAMs to the M2-like phenotype associated a pro-tumour function. Taken together, I hypothesised that conditioned media from FAK-wt tumours would polarise naïve $CD4^+$ T-cell to the T_H2 phenotype. $CD4^+$ T-cells derived from splenocytes from C57BL/6 mice were isolated using CD4 microbeads, cultured in either CM derived from FAK-wt or FAK-/- tumour cell lines and harvested after 24, 48 72 and 96 hours. Cells were disaggregated and analysed using an intracellular transcription staining panel to identify T_H2 cells (**stain 4, table 6.2**). The percentage of $CD4^+$ T-cells expressing GATA3, a transcription factor used to identify T_H2 cells, increases in a time dependent manner in $CD4^+$ T-cells cultured in Panc47 FAK-wt CM and is expressed at barely detectable levels in $CD4^+$ T-cells cultured in Panc47 FAK-/- CM. These results show that Panc47 FAK-wt CM is sufficient to induce expression of a hallmark transcription factor that drives differentiation of $CD4^+$ T-cell to the T_H2 phenotype, providing the first evidence that FAK may regulate the T_H1/T_H2 balance within the pancreatic TME.

STAIN 4	
MARKER	**FLUOROPHORE**
Viability	Zombie NIR
CD4	BV650
GATA3	PE

Table 6.2 Flow cytomety transcription panel used to identify T_H2 (GATA3 positive $CD4^+$ T-cells)

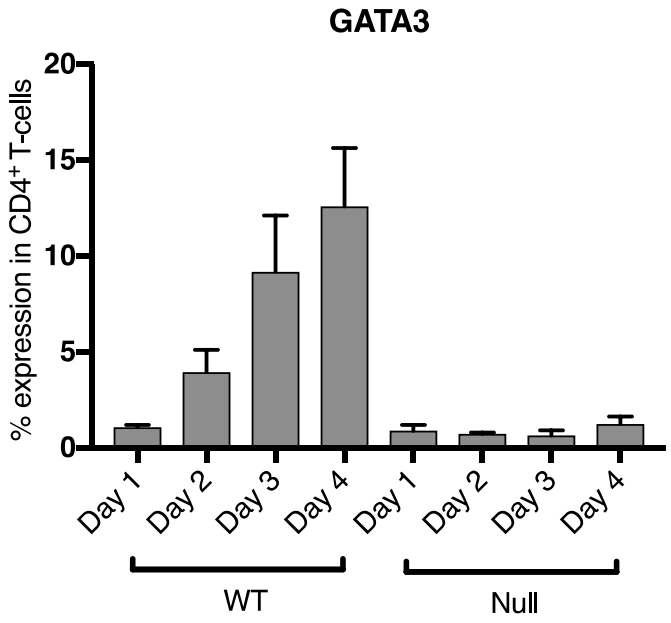


Figure 6.3 Effects of FAK-wt and FAK-/- CM on GATA3 expression of naïve $CD4^+$ T-cells

$CD4^+$ T-cells were isolated with CD4 microbeads using the autoMACS® Pro Separator from splenocytes derived from C57BL/6 mice. Cells were seeded at 2 x 10^5 cells in a 48 well plate and cultured in 1ml of FAK-wt or FAK-/- CM supplemented with an extra 10% FCS, then harvested after 24, 48 72 and 96 hours. (Figure courtesy of Dr David Taggart**)** GATA3 was measured with flow cytometry and is expressed as percentage of $CD4^+$. Results represent n=3, error bars represent SEM

6.3 Discussion

The *in-vitro* induction of macrophages to a pro-tumour associated phenotype by IL6 was found to require the addition of IL4. A likely source of IL4 in the PDAC TME is T_H2 polarised $CD4^+$ T-cells. In support of a potential role for these cells in regulating the macrophage phenotype in Panc47 FAK-wt tumours, I identified that $CD4^+$ T-cells were required for FAK-mediated PDAC growth acceleration and induction of the pro-tumour associated macrophage phenotype *in-vivo*. IL4 is not produced at detectable levels by Panc47 FAK-wt cells leading me to conclude that IL6 mediated induction of MMR and PD-L2 on TAMs requires the addition of IL4, which likely come from $CD4^+$ T-cells. IL6 is known to induce T_H2 cells. FAK-wt conditioned media induced naïve $CD4^+$ T-cells to the T_H2 phenotype and I propose that FAK-regulated IL6 derived from pancreatic tumour cells drives this phenotypic switch. Further *in-vitro* work is required to confirm that FAK-dependent IL6 specifically drives this regulation and additional *in-vivo* analysis is necessary to establish an increased presence of T_H2 cells in FAK-wt tumours compared to FAK-/- tumours. Nevertheless I show that FAK drives a hallmark of T_H2 T-cells, the first evidence to suggest FAK may shift the T_H1/T_H2 TME balance towards T_H2.

Whilst IL6 alone or Panc47 FAK-wt conditioned media induced a moderate expression of MMR in BMDM *in-vitro*, this was enhanced by the addition of IL4. However IL6 alone or Panc47 FAK-wt conditioned media minimally induced expression of PD-L2, but a moderate increase in expression was induced by IL4 alone. This was enhanced by the addition of IL6 or Panc47 FAK-wt CM. I therefore conclude from this that both IL4 and IL6 are important in up-regulation of MMR and PD-L2, but are perhaps not working though the same mechanism. Indeed, up-regulation of PD-L2 is thought to be a Stat-6 dependent process whereas MMR expression is independent of Stat-6[399].

T_H2-type inflammation facilitates tumour growth[358] and as such, the presence of T_H2 cells are a negative indicator of patient survival in pancreatic cancer[99]. IL4 plays a key role in the T_H2 immune response, serving as both an initiator and effector of T_H2 immune reactions[344]. Increased IL4 is associated with increased pancreatic cancer growth and invasion[401] and the predominant source of IL4 within the tumour microenvironment is T_H2 polarised $CD4^+$ T-cells[402]. When $CD4^+$ T-cells were

depleted in mice, there was a significant reduction in tumour size and reduced expression of pro-tumour associated macrophage markers in Panc47 FAK-wt tumours. Further, the requirement of IL4 in polarising macrophages to the pro-tumour associated phenotype *in-vitro* substantiates the importance of T_H2 cells in this mechanism.

No significant difference in tumour weight was observed between Panc47 FAK-wt tumours from mice treated with $CD4^+$ T-cell depleting antibody and Panc47 FAK-/- tumours treated with either isotype control or $CD4^+$ depleting antibody, although Panc47 FAK-wt tumours from mice treated with $CD4^+$ T-cell depleting antibody were in general slightly heavier. This is perhaps not surprising given the pleotropic role FAK plays in regulating multiple cellular processes that contribute to tumour growth, and these results further support a contribution from FAK regulated immune independent mechanisms in the growth delay of FAK-/- tumours.

IL6 was also shown to be crucial to FAK-dependent polarisation of macrophages *in-vitro* (**figure 6.2**) and *in-vivo* (**chapter 5**), in addition to acceleration of tumour growth (**chapter 5**). The additional requirement of IL4 and $CD4^+$ T-cells suggests that this is both a direct and an indirect mechanism. IL6 is known to induce naïve $CD4^+$ T-cell to the T_H2 ($GATA3^+$) phenotype. The T_H2 marker GATA3 is associated with rapid disease progression and diminished overall patient survival in pancreatic cancer[99]. During antigen stimulation of naïve $CD4^+$ T-cells, IL6 induces autocrine, self-sustaining IL4 production in $CD4^+$ T-cells[94,403] and inhibits expression of the T_H1 cytokine IFNγ[95]. Conditioned media from Panc47 FAK-wt cells increased $GATA3^+$ $CD4^+$ T-cells, indicating a switch to the T_H2 phenotype. Further *in-vitro* work is required to establish whether FAK-dependent IL6 specifically drives this regulation, and further *in-vivo* analysis will be required to confirm an increased presence of T_H2 cells in Panc47 FAK-wt tumours compared to FAK-/- tumours. Nevertheless I show that FAK drives a hallmark of T_H2 T-cells, the first evidence to suggest FAK may shift the T_H1/T_H2 TME balance towards T_H2.

7 General Discussion

7.1 Principle findings

- FAK expression in pancreatic cancer accelerates tumour growth by suppressing an anti-tumour $CD8^+$ T-cell response.
- FAK expression in pancreatic cancer results in reprogramming of macrophages within the tumour microenvironment to a phenotype associated with a pro-tumour response.
- FAK-dependent regulation of the macrophage phenotype and pancreatic tumour growth is dependent on $CD4^+$ T-cells and cancer cell derived IL6, a FAK-regulated cytokine requiring FAK-kinase activity and nuclear FAK localisation.
- Expression of MMR and PD-L2 on macrophages is driven by the cooperative action of IL6 and IL4.
- The secretome of Panc47 FAK-wt pancreatic cancer cells can polarise $CD4^+$ T-cells towards a T_H2 phenotype.

7.2 General discussion and therapeutic implications

I show that pancreatic cancer cell intrinsic FAK signalling plays an important role in regulating pancreatic tumour growth through promoting evasion of the $CD8^+$ T-cell anti-tumour immune response, and that this is associated with regulation of the tumour associated macrophage (TAM) phenotype. Specifically I identify that FAK-depletion in pancreatic cancer cells shifts the balance from $Ly6C^+MMR^+CD86^-$ macrophages towards $Ly6C^+MMR^-CD86^+$ macrophages, a change that is frequently associated with acquisition of an anti-tumour phenotype[59]. Further, I also observe a significant down-regulation of PD-L2 expression on the surface of all TAMs in FAK-depleted pancreatic tumours, implying that FAK-dependent paracrine signalling between cancer cells and macrophages can regulate macrophage immunosuppressive function. Mechanistically, I identify FAK-dependent regulation of IL6 secretion from pancreatic cancer cells as a key pathway in controlling both

pancreatic tumour growth and the TAM phenotype, and further show that this requires a contribution from tumour infiltrating $CD4^+$ T-cells, suggesting a complex multicellular mechanism that is driven by FAK-dependent IL6 secretion. Using bone marrow derived macrophage cultures, I establish that the macrophage phenotype observed in Panc47 FAK-wt tumours can be recapitulated with a combination of IL6 and IL4, but not either cytokine alone. Furthermore, naïve $CD4^+$ T-cells cultured in the presence of Panc47 FAK-wt or FAK-/- tumour cell conditioned media (CM) identified that only Panc47 FAK-wt CM drives expression of the transcription factor GATA3 in $CD4^+$ T-cells. This implies that the secretome of Panc47 FAK-wt pancreatic cancer cells can polarise $CD4^+$ T-cells towards a T_H2 phenotype, a phenotype typically associated with expression of IL4. Therefore, I report a previously unknown mechanism through which pancreatic cancer cell intrinsic FAK signalling drives expression of IL6 and T_H2 polarisation of $CD4^+$ T-cells in order to reprogram the TAM phenotype and promote pancreatic tumour growth and immune suppression (summarised in **figure 7.1**).

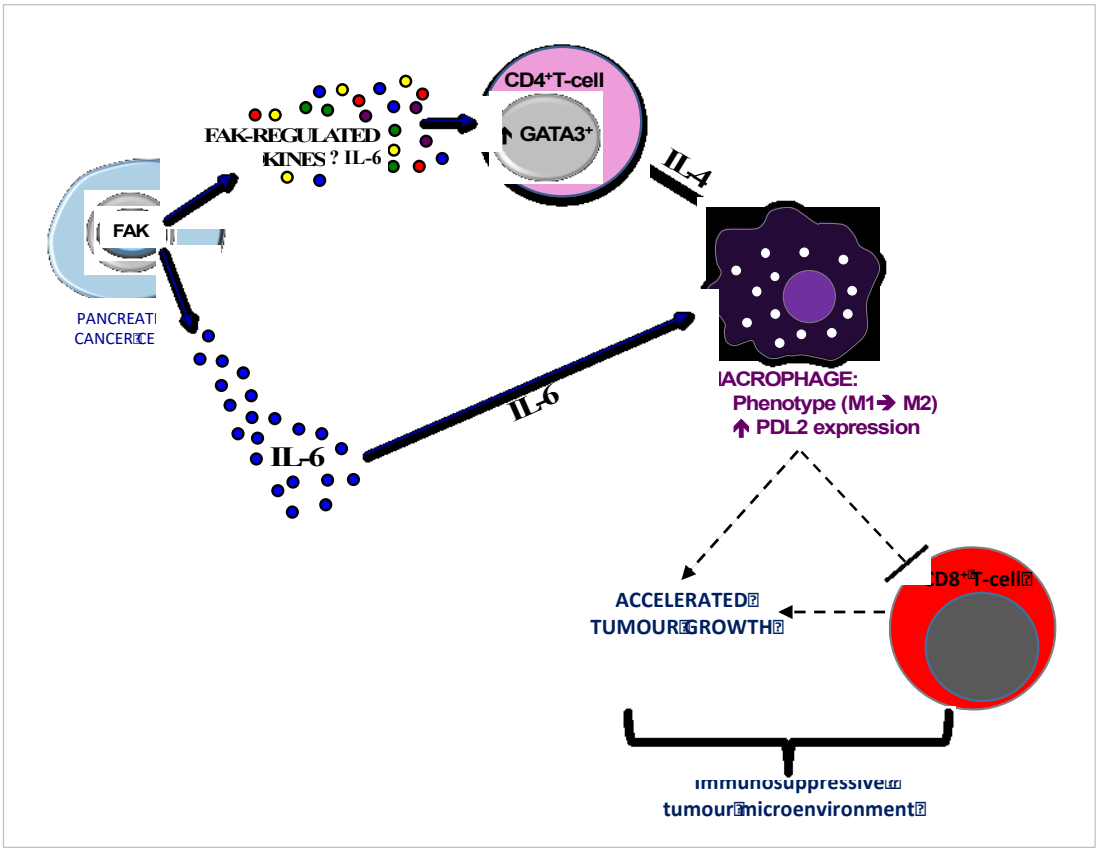


Figure 7.1 FAK signalling in pancreatic cancer cells regulates mechanisms of immune evasion to promote tumour growth

FAK-regulated cytokines secreted by pancreatic cancer cells initiates induction of naïve $CD4^+$ T-cells to the T_H2 phenotype. IL4 most likely produced by T_H2 cells acts in combination with cancer cell derived IL6 to reprogram tumour associated macrophages to a phenotype associated with tumour promotion, thus creating an immunosuppressive tumour microenvironment, with inhibition of cytotoxic T-cells and acceleration of tumour growth.

Macrophages are the most abundant immune cell type within the pancreatic TME, and a number of previous studies have established an important role for these cells in promoting pancreatic tumour development and progression, through regulating invasion, angiogenesis, matrix remodelling, metastasis, immunosuppression and suppression of the $CD8^+$ T-cell anti-tumour immune response[42,56,60,404]. For this reason they have become an attractive therapeutic target for the treatment of cancer and a number of experimental therapeutics targeting macrophage recruitment and / or function are currently in clinical testing including CCR2 and CSF1R inhibitors (clinicaltrials.gov NCT01413022, NCT03184870, NCT02452424, NCT02829723, NCT01316822, NCT01346358). However, these therapeutic approaches do not

specifically target tumour associated macrophage populations, rather they target all macrophages, and as a consequence may perturb the homeostatic function of macrophages within other tissues of the body, leading to unwanted immune-mediated side-effects. Similar problems blight many current immunotherapy approaches, highlighting a need to identify more tumour-specific approaches for targeting immune cell function. FAK, in particular neoplastic cell intrinsic FAK signalling, is emerging as an important regulator of the immunosuppressive TME in cancer. In a mouse model of skin SCC, FAK-dependent expression of the chemokine CCL5 was shown to drive increased Treg recruitment to tumours, resulting in suppression of the anti-tumour $CD8^+$ T-cell response and tumour survival[197]. Importantly, this was mediated by nuclear FAK signalling which does not appear to be prevalent in normal cells, suggesting that FAK-mediated immune regulation may have an element of tumour specificity. Similar findings demonstrating FAK's role in general regulation of the immunosuppressive TME have since been reported in mouse models of pancreatic cancer. Treatment with a FAK kinase inhibitor resulted in broad reprograming of the immunosuppressive TME and sensitization to a combination of chemotherapy (Gemcitabine) and immune checkpoint blockade (anti-PD-1 + anti-CTLA4)[142]. Here I show that FAK signalling in pancreatic cancer cells can regulate the macrophage phenotype in pancreatic tumours through control of a paracrine signalling axis that requires the cytokine IL6, a cytokine regulated by nuclear FAK in a kinase-dependent manner.

Specifically, I found that this axis enriches for MMR^+CD86^- macrophages in the $Ly6C^+$ population. Ly6C marks a population of inflammatory or immature macrophages undergoing differentiation from bone marrow derived monocytes into mature macrophages[405,406], and MMR is typically associated with a pro-tumour phenotype[407,408]. Therefore, these findings suggest that FAK, at least in part through the action of IL6, reprograms macrophages during differentiation to adopt a more pro-tumour phenotype. The lack of MMR regulation in $Ly6C^-$ macrophages also suggests a distinct mechanism of regulation following differentiation that is not dependent on the action of IL6. I additionally identified that FAK loss in pancreatic cancer cells results in a reduction in the expression of the immune checkpoint ligand PD-L2 in all macrophage populations, and that this was also dependent on IL6

signalling. PD-L2 blockade has been shown to reduce the ability of macrophages to inhibit cytotoxic T-cell proliferation[409] and expression is thought to be a Stat-6 dependent process. In contrast MMR expression is independent of Stat-6[399,409], supporting my findings that MMR and PDL2 are likely regulated via distinct mechanisms. There is an increasing appreciation that macrophages display a high amount of phenotypic plasticity and different sub-populations are likely to have different functions and/ or origins in the cancer TME, which may differ between cancer types[357]. This also may provide an alternative explanation for the differential regulation of FAK-induced receptor/ ligand expression. Based on separation of populations seen on flow cytometry analysis, we identified four different sub-populations of macrophages: ($CD45^+CD11b^{hi}F480^+Ly6C^+$, $CD45^+CD11b^{hi}F480^+Ly6C$, $CD45^+CD11b^{lo}F480^+Ly6C^+$ and $CD45^+CD11b^{lo}F480^+Ly6C^-$). Separation and further *in-vitro* functional assays of these identified populations were not performed, therefore we can only speculate on differences between FAK-induced receptor/ ligand expression and function between these subpopulations. However in addition to potentially differing functions, these subpopulations may also have different origins. There are conflicting reports in the literature regarding the origin of TAMs. Several studies suggest that most TAMs arise from the $Ly6C^+$ population of circulating monocytes, for example in mouse models of mammary tumours[357,410], and in murine lung metastases[411]. However recent reports demonstrate that tissue resident macrophages in the normal pancreas are embryonically derived[346], and that in pancreatic cancer, both these embryonically derived tissue resident macrophages as well as infiltrating bone marrow derived macrophages contribute to the TAM population. Lineage tracing experiments were not performed but would be an interesting series of follow up experiments. Both of these populations increase in density as the tumour progresses[56]. *In-vitro* analysis of BMDM receptor expression demonstrated differing responses to cytokine exposure. IL6 alone or Panc47 FAK-wt conditioned media induced a moderate expression of MMR in BMDM *in-vitro*, and this was enhanced by the addition of IL4. However IL6 alone or Panc47 FAK-wt conditioned media minimally induced expression of PD-L2 and a moderate increase in expression was induced by IL4 alone. This was enhanced by the addition of IL6 or Panc47 FAK-wt CM. Notably, the vast majority of BMDMs were $Ly6C^+$ (data not shown). IL4 induces alternative activation of

macrophages in both embryonically or monocyte-derived populations regardless of origins[412] but IL4 induced up-regulation of PD-L2 is limited to monocyte-derived macrophages[399]. My *in-vitro* macrophage polarisation experiments were conducted on BMDMs, which may represent only the monocyte-derived populations of the TAMs found within the PDAC TME. It would therefore be interesting to FACS sort macrophages from implanted Panc47 FAK-wt and FAK-/- tumours grown *in-vivo* and repeat these stimulation experiments and re-assess the resultant surface receptor expression.

IL6 is emerging as an important cytokine in the development and progression of pancreatic cancer. Recent evidence has uncovered the role of cancer cell derived IL6 in immune evasion and its role in shaping immune cell populations such as dendritic cells, macrophages and T-cells within the TME, disabling both the innate and adaptive immune system. Increased IL6 pathway signalling is thought to be in part responsible for the T_H2 polarised TME and impaired T_H1 response found in cancer[96,413]. Indeed inhibition of the IL6 receptor has been found to improve T_H1 development resulting in an antitumor response[92] and IL6 inhibition in ovarian cancer patients, combined with interferon treatment, has recently demonstrated some treatment success in reversing cancer associated immunosuppression with increased tumour anti-tumour $CD8^+$ T-cell infiltration[414]. IL6 has also been linked to age related intrinsic T-cell dysfunction and decreased susceptibility to cancer immunotherapies[96]. As elderly people represent the vast majority of cancer patients, control of IL6 levels should perhaps be taken into account in a comprehensive treatment strategy in cancer therapy. Therefore treatment strategies targeting IL6 may indirectly impact downstream immunosuppressive targets as well as cytotoxic T-cell dysfunction. This new knowledge makes strategies targeting IL6 in combination with current immunotherapies appealing and as such IL6 inhibition has already been shown to improve responses to immunotherapies in pre-clinical models[97,98]. I show FAK-dependent regulation of IL6 secretion from pancreatic cancer cells regulates both pancreatic tumour growth and the TAM phenotype, in agreement with previous reports demonstrating the role of IL6 in pro-tumour macrophage polarisation[176,415]. FAK inhibition may therefore represent a novel avenue for control of IL6 within the PDAC TME, in addition to a plethora of other

anti-cancer benefits that FAK inhibition brings such as blockage of VEGF production and reduced micro-vessel density, reduced tumour cell survival and proliferation, reduced migration and invasion, amongst other benefits[267,416].

However, my results suggest that IL6 does not function alone, but rather cooperates with IL4, and likely other cytokines, to establish a tumour promoting and immune suppressive TME. I found that IL6 was not sufficient to induce the phenotype of macrophages identified in Panc47 FAK-wt tumours when applied to bone marrow derived macrophage cultures *in-vitro*. Rather, it required the addition of IL4 to fully recapitulate the macrophage phenotype. However, analysis of the cytokine secretion from Panc47 FAK-wt tumour cells did not identify any IL4 secretion, implying that it must come from a different source within the TME. Further functional studies identified that $CD4^+$ T-cells were required to promote Panc47 FAK-wt tumour growth, and that $CD4^+$ T-cell depletion resulted in a reduction of MMR^+CD86^- macrophages in the $Ly6C^+$ population and a reduction in PD-L2 expression across all macrophage subsets, similar to the results found with FAK-depletion. Thus there is a requirement of an accessory cell type in this mechanism, most likely the T_H2 polarised $CD4^+$ T-cell. This highlights the complexity of the pancreatic TME and it is likely that multiple cell types are recruited and cell phenotypes induced, which may vary between patients and in temporal fashion. Additionally, downstream effects of tumour-derived cytokines such as IL6 may not be limited to the local effects influencing the TME. Tumour cell production of IL6[417] and also G-CSF and GM-CSF can induce bone marrow myeloid progenitor expansion, leading to increased myeloid cells in the circulation and resultant infiltration into tumours and disease progression[418,419]. Furthermore, in KPC murine PDAC models, tumour-derived IL6 has been shown to alter liver metabolism and consequently, in the context of caloric restriction, leads to increased corticosterone production and suppressed antitumor immunity[420]. Therefore it may be that cytokines derived from the tumour site are working in complex, multimodal, local and systemic mechanisms. Understanding the individual complexities unique to the TME of individual cancer types is imperative in the selection of therapies designed to treat different cancer types. Multiple arms of cancer-induced immunosuppression must be addressed to avoid some of the pitfalls in currently available immunotherapies and also in the

development of novel therapeutic approaches to target aspects of the immunosuppressive TME and reverse immune evasion.

There is an increasing appreciation that T_H2 polarised TMEs are important in the context of cancer. Specifically in pancreatic cancer, T_H2 cells are negative indicators of patient survival[99]. Therapeutic strategies targeting the T_H2 weighted environment will likely be required to effectively target M2 TAMS and address cancer immune evasion. The pro-tumour M2 TAM phenotype is induced not only by IL6[176], but also by IL4[399] and are more generally promoted in a T_H2 weighted TME, a TME that is known to favour pancreatic tumour progression[47]. Further to FAK's regulation of IL6, I show the first evidence demonstrating that FAK may shift the T_H1/T_H2 TME balance towards T_H2. IL6 is a component of Panc47 FAK-wt CM, which I show polarises naïve $CD4^+$ T-cells to the T_H2 ($GATA3^+$) phenotype. I show that cancer cell derived IL6 polarises BMDM to the M2/ pro-tumour associated phenotype but requires the addition of IL4, a cytokine secreted by T_H2 cells. This novel function of FAK corroborates previous reports demonstrating the requirement of IL4 in skewing TAMs toward the pro-tumorigenic M2 phenotype in mammary cancer[421], the requirement of IL6 in promoting the pro-tumour M2 phenotype in TAMs in lung cancer[176] and IL6 mediated T_H1 inhibition[92].

I set out to identify novel mechanisms of FAK-dependent immune regulation in pancreatic cancer. The result of my research establishes FAK as an important regulator of the immunosuppressive environment within pancreatic cancer via a multicellular mechanism that is driven by FAK-dependent secretion of IL6. Thus this provides new insights into the complexity of FAK-dependent immune regulation in pancreatic cancer, and supports further investigation of FAK inhibitors in combination with other immunotherapy treatments for the management of PDAC.

7.3 Future work

One pitfall of using discrete flow cytometry marker panels to define macrophage subpopulations and to infer functional changes within the pancreatic TME is that this approach may oversimplify the range of phenotypes present within the TME and the varying functions of these cells. Although this provides a foundation for future efforts, deeper analysis of the complexity of cells within the pancreatic TME, using

technologies such as high- resolution single-cell RNA sequencing and intra-vital imaging, will provide exceptionally detailed information on the polarisation status, composition, function and spatial distribution of cells such as macrophages with in the PDAC TME. The information gained from a deeper understanding of the complexity of the TME through improved resolution of the cellular composition could also prove useful in identifying novel therapeutic targets and also in the individualised medicine arena, if paired with relevant patient outcomes, and may better identify patients who may derive benefit from these therapies. Single cell RNA sequencing is currently underway to examine the macrophage populations within Panc47 tumours in finer detail.

Future work will also focus on further defining FAK's role in polarising $CD4^+$T-cells to the T_H2 phenotype. This will include further *in-vitro* assays comparing $CD4^+$ T-cell polarisation using Panc47 FAK-wt IL6 shRNA1/2 and the FAK-/- IL6 lenti re-expression lines to determine if cancer cell derived IL6 is specifically involved in T_H2 polarisation, which will expand on the work already completed comparing Panc47 FAK-wt and FAK-/- CM on GATA3 expression. Additionally, we will compare levels of T_H2 cells between FAK-wt and FAK-/- tumours. There are a variety of ways of conducting these experiments *in-vivo,* either with use of flow cytometry with specific transcription and intracellular cytokine panels, or with histology using GATA3, STAT5 and CD4 co-staining. It would additionally be interesting to utilise the inducible IL4 reporter mouse to ascertain whether there are higher numbers of $CD4^+$ T-cells within FAK-wt tumours compared to FAK-/- tumour

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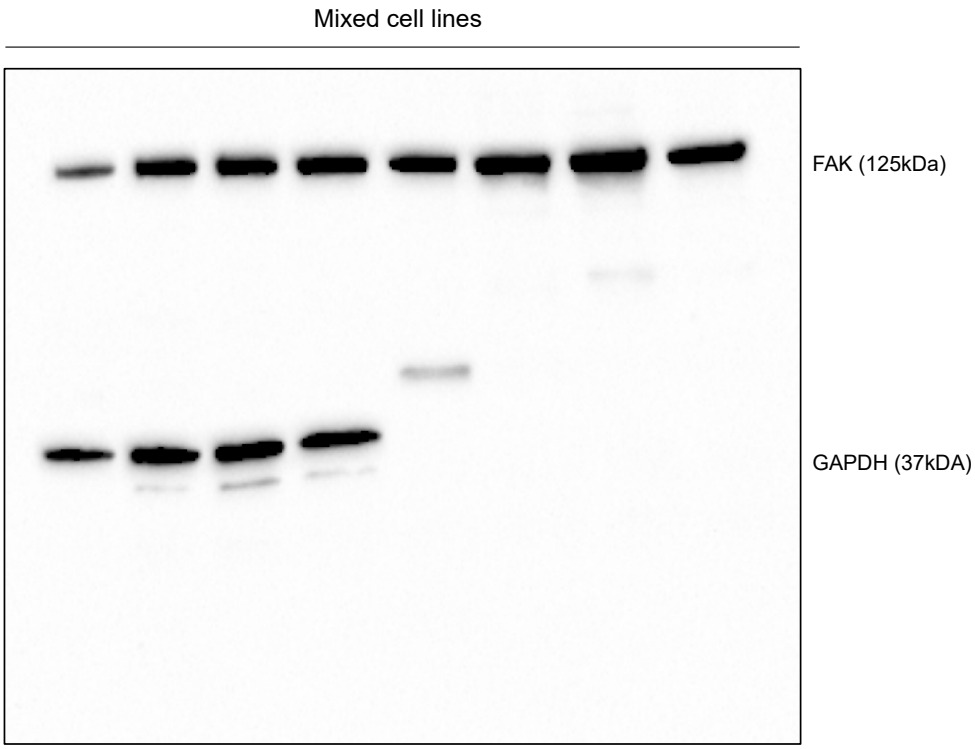
9 Appendices

9.1 Appendix 1: Full western blot of parental PDAC cell line (Panc47)



Full western blot of parental PDAC cell line (Panc47) corresponding to **figure 3.2**. Western blot demonstrates whole cell, nuclear and cytoplasmic fractions probed with antibodies specific to total FAK and GAPDH. Lane 2 contains whole cell Panc47. Lane 4 contains Panc47 cytoplasmic portion and lane 5 contains Panc47 nuclear fraction. Remaining lanes contain other cell lines.

9.2 Appendix 2: Comparison of human, chicken and mouse FAK DNA sequence

```
Chicken_FAK     MAAAYLDPNLNHTPSSSAKTHLGTGMERSPGAMERVLKVFHYFENSSEPTTWASIIRHGD   60
Mouse-FAK       MAAAYLDPNLNHTPSSSTKTHLGTGMERSPGAMERVLKVFHYFESSSEPTTWASIIRHGD   60
Human_FAK       MAAAYLDPNLNHTPNSSTKTHLGTGMERSPGAMERVLKVFHYFESNSEPTTWASIIRHGD   60
                **************.**:*************************..**************

Chicken_FAK     ATDVRGIIQKIVDCHKVKNVACYGLRLSHLQSEEVHWLHLDMGVSNVREKFELAHPPEEW   120
Mouse-FAK       ATDVRGIIQKIVDSHKVKHVACYGFRLSHLRSEEVHWLHVDMGVSSVREKYELAHPPEEW   120
Human_FAK       ATDVRGIIQKIVDSHKVKHVACYGFRLSHLRSEEVHWLHVDMGVSSVREKYELAHPPEEW   120
                *************.****:*****:*****:********:*****.****:*********

Chicken_FAK     KYELRIRYLPKGFLNQFTEDKPTLNFFYQQVKNDYMLEIADQVDQEIALKLGCLEIRRSY   180
Mouse-FAK       KYELRIRYLPKGFLNQFTEDKPTLNFFYQQVKSDYMQEIADQVDQEIALKLGCLEIRRSY   180
Human_FAK       KYELRIRYLPKGFLNQFTEDKPTLNFFYQQVKSDYMLEIADQVDQEIALKLGCLEIRRSY   180
                *******************************.*** **********************

Chicken_FAK     GEMRGNALEKKSNYEVLEKDVGLRRFFPKSLLDSVKAKTLRKLIQQTFRQFANLNREESI   240
Mouse-FAK       WEMRGNALEKKSNYEVLEKDVGLKRFFPKSLLDSVKAKTLRKLIQQTFRQFANLNREESI   240
Human_FAK       WEMRGNALEKKSNYEVLEKDVGLKRFFPKSLLDSVKAKTLRKLIQQTFRQFANLNREESI   240
                 **********************:*****************************************

Chicken_FAK     LKFFEILSPVYRFDKECFKCALGSSWIISVELAIGPEEGISYLTDKGANPTHLADFNQVQ   300
Mouse-FAK       LKFFEILSPVYRFDKECFKCALGSSWIISVELAIGPEEGISYLTDKGCNPTHLADFNQVQ   300
Human_FAK       LKFFEILSPVYRFDKECFKCALGSSWIISVELAIGPEEGISYLTDKGCNPTHLADFTQVQ   300
                ***********************************************.********.***

Chicken_FAK     TIQYSNSEDKDRKGMLQLKIAGAPEPLTVTAPSLTIAENMADLIDGYCRLVNGATQSFII   360
Mouse-FAK       TIQYSNSEDKDRKGMLQLKIAGAPEPLTVTAPSLTIAENMADLIDGYCRLVNGATQSFII   360
Human_FAK       TIQYSNSEDKDRKGMLQLKIAGAPEPLTVTAPSLTIAENMADLIDGYCRLVNGTSQSFII   360
                ************************************************::*****

Chicken_FAK     RPQKEGERALPSIPKLANNEKQGVRSHTVSV----------------------------   391
Mouse-FAK       RPQKEGERALPSIPKLANSEKQGMRTHAVSVSHCQHKVKKARRFLPLVFCSLEPPPTDEI   420
Human_FAK       RPQKEGERALPSIPKLANSEKQGMRTHAVSVS---------------------------   392
                *****************.****:*:*:***

Chicken_FAK     --SETDDYAEIIDEEDTYTMPS-------TRDYEIQRERIELGRCIGEGQFGDVHQGIYM   442
Mouse-FAK       SGDETDDYAEIIDEEDTYTMPSKSYGIDEARDYEIQRERIELGRCIGEGQFGDVHQGVYL   480
Human_FAK       ---ETDDYAEIIDEEDTYTMPS-------TRDYEIQRERIELGRCIGEGQFGDVHQGIYM   442
                   ******************       :***************************:*:

Chicken_FAK     SPENPAMAVAIKTCKNCTSDSVREKFLQEALTMRQFDHPHIVKLIGVITENPVWIIMELC   502
Mouse-FAK       SPENPALAVAIKTCKNCTSDSVREKFLQEALTMRQFDHPHIVKLIGVITENPVWIIMELC   540
Human_FAK       SPENPALAVAIKTCKNCTSDSVREKFLQEALTMRQFDHPHIVKLIGVITENPVWIIMELC   502
                ******:*********************************************************

Chicken_FAK     TLGELRSFLQVRKFSLDLASLILYAYQLSTALAYLESKRFVHRDIAARNVLVSATDCVKL   562
Mouse-FAK       TLGELRSFLQVRKYSLDLASLILYAYQLSTALAYLESKRFVHRDIAARNVLVSSNDCVKL   600
Human_FAK       TLGELRSFLQVRKYSLDLASLILYAYQLSTALAYLESKRFVHRDIAARNVLVSSNDCVKL   562
                *************:*****************************************:.*****

Chicken_FAK     GDFGLSRYMEDSTYYKASKGKLPIKWMAPESINFRRFTSASDVWMFGVCMWEILMHGVKP   622
Mouse-FAK       GDFGLSRYMEDSTYYKASKGKLPIKWMAPESINFRRFTSASDVWMFGVCMWEILMHGVKP   660
Human_FAK       GDFGLSRYMEDSTYYKASKGKLPIKWMAPESINFRRFTSASDVWMFGVCMWEILMHGVKP   622
                ************************************************************

Chicken_FAK     FQGVKNNDVIGRIENGERLPMPPNCPPTLYSLMTKCWAYDPSRRPRFTELKAQLSTILEE   682
Mouse-FAK       FQGVKNNDVIGRIENGERLPMPPNCPPTLYSLMTKCWAYDPSRRPRFTELKAQLSTILEE   720
Human_FAK       FQGVKNNDVIGRIENGERLPMPPNCPPTLYSLMTKCWAYDPSRRPRFTELKAQLSTILEE   682
                ************************************************************

Chicken_FAK     EKLQQEERMRMESRRQVTVSWDSGGSDEAPPKPSRPGYPSPRSSEGFYPSPQHMVQPNHY   742
Mouse-FAK       EKVQQEERMRMESRRQATVSWDSGGSDEAPPKPSRPGYPSPRSSEGFYPSPQHMVQTNHY   780
Human_FAK       EKAQQEERMRMESRRQATVSWDSGGSDEAPPKPSRPGYPSPRSSEGFYPSPQHMVQTNHY   742
                ** *************.****************************************** ***

Chicken_FAK     QVSGYSGSHGIPAMAGSIYPGQASLLDQTDSWNHRPQEVSAWQPNMEDSGTLDVRGMGQV   802
Mouse-FAK       QVSGYPGSHGIPAMAGSIYQGQASLLDQTELWNHRPQEMSMWQPSVEDSAALDLRGMGQV   840
Human_FAK       QVSGYPGSHGITAMAGSIYPGQASLLDQTDSWNHRPQEIAMWQPNVEDSTVLDLRGIGQV   802
                ***** ***** ******* *********: *******:: ***.:*** .**:**:***

Chicken_FAK     LPTHLMEERLIRQQQEMEEDQRWLEKEERFLVMKPDVRLSRGSIEREDGGLQGPAGNQHI   862
Mouse-FAK       LPPHLMEERLIRQQQEMEEDQRWLEKEERFL--KPDVRLSRGSIDREDGSFQGPTGNQHI   898
Human_FAK       LPTHLMEERLIRQQQEMEEDQRWLEKEERFL--KPDVRLSRGSIDREDGSLQGPIGNQHI   860
                ** ************************  ***********:****.:*** *****

Chicken_FAK     YQPVGKPDHAAPPKKPPRPGAP-HLGSLASLNSPVDSYNEGVKIKPQEISPPPTANLDRS   921
Mouse-FAK       YQPVGKPDPAAPPKKPPRPGAPGHLSNLSSISSPADSYNEGVKLQPQEISPPPTANLDRS   958
Human_FAK       YQPVGKPDPAAPPKKPPRPGAPGHLGSLASLSSPADSYNEGVKLQPQEISPPPTANLDRS   920
                ******** ************ **..*:*:.**.********::*****************

Chicken_FAK     NDKVYENVTGLVKAVIEMSSKIQPAPPEEYVPMVKEVGLALRTLLATVDESLPVLPASTH   981
Mouse-FAK       NDKVYENVTGLVKAVIEMSSKIQPAPPEEYVPMVKEVGLALRTLLATVDETIPALPASTH   1018
Human_FAK       NDKVYENVTGLVKAVIEMSSKIQPAPPEEYVPMVKEVGLALRTLLATVDETIPLLPASTH   980
                ************************************************::* ******

Chicken_FAK     REIEMAQKLLNSDLAELINKMKLAQQYVMTSLQQEYKKQMLTAAHALAVDAKNLLDVIDQ   1041
Mouse-FAK       REIEMAQKLLNSDLGELISKMKLAQQYVMTSLQQEYKKQMLTAAHALAVDAKNLLDVIDQ   1078
Human_FAK       REIEMAQKLLNSDLGELINKMKLAQQYVMTSLQQEYKKQMLTAAHALAVDAKNLLDVIDQ   1040
                **************.***.****************************************

Chicken_FAK     ARLKMISQSRPH   1053
Mouse-FAK       ARLKMLGQTRPH   1090
Human_FAK       ARLKMLGQTRPH   1052
                *****:.*:***
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